Vectors, Mutant Viruses and Methods for Generating Mutant Viruses

Field of the Invention

The present invention relates to nucleic acid vectors for delivery of a nucleic acid cassette to an insertion site in a selected viral genome, to methods of generating mutant virus using said vectors and to the mutant viruses generated, and particularly, although not exclusively, to mutant herpes simplex viruses and nucleic acid vectors for use in generating mutant herpes simplex viruses.

Background to the Invention

Existing procedure for generating herpes simplex virus (HSV) mutants requires generation of a unique plasmid by cloning an entire expression cassette consisting of a promoter, gene of interest and polyadenylation sequences into a plasmid separately constructed to contain the relevant flanking sequences and then co-transfecting BHK cells with the resultant plasmid and HSV-1 DNA. Homologous recombination drives the formation of recombinant HSV-1 expressing the gene of interest, which is identified by Southern blotting. The recombinant virus is plaque purified 3-4 times by Southern blotting. This process takes several months.

This approach was taken by Liu et al[1] in generating two distinct plasmids, the first consisting of HSV-1 strain 17+ Sau3A fragment derived sequences flanking an expression cassette consisting of a CytoMegalovirus (CMV)

promoter, Green Fluorescent Protein (GFP) gene and bGH polyadenylation (polyA) signal and the second wherein the GFP gene is replaced with either a human or mouse Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) gene.

Shuttle vectors have been used to generate recombinant adenoviral vectors, e.g. the pAdEasy™ system of vectors (Stratagene), for use in overexpressing recombinant proteins in mammalian cells. However, these vectors require the cloning of the gene of interest into a first shuttle vector which is then co-transformed into a specially constructed cell line to generate a recombinant adenoviral plasmid which is transfected into a separate specially constructed mammalian cell line in which the recombinant adenoviral plasmid is directly packaged into virus particles.

The HSV genome comprises two covalently linked segments, designated long (L) and short (S). Each segment contains a unique sequence flanked by a pair of inverted terminal repeat sequences. The long repeat (RL or R_L) and the short repeat (RS or R_S) are distinct.

The HSV ICP34.5 (also γ34.5) gene, which has been extensively studied[1,6,7,8], has been sequenced in HSV-1 strains F[9] and syn17+[3] and in HSV-2 strain HG52[4]. One copy of the ICP34.5 gene is located within each of the RL repeat regions. Mutants inactivating both copies of the ICP34.5 gene (i.e. null mutants), e.g. HSV-1 strain 17 mutant 1716[2] (HSV1716) or the mutants R3616 or R4009 in strain F[5], are known to lack neurovirulence, i.e. be

avirulent, and have utility as both gene delivery vectors or in the treatment of tumours by oncolysis. HSV1716 has a 759bp deletion in each copy of the ICP34.5 gene located within the BamHI s restriction fragment of each RL repeat.

ICP34.5 null mutants such as HSV1716 are, in effect, first-generation oncolytic viruses. Most tumours exhibit individual characteristics and the ability of a broad spectrum first generation oncolytic virus to replicate in or provide an effective treatment for all tumour types is not guaranteed.

The prior art provides technically challenging, procedurally slow and inefficient materials and methods for generating recombinant HSV. In particular the prior art does not provide methods of, and materials for, generating recombinant HSV which are easy to detect, may be designed to be specific null mutants and which may express a selected gene of interest.

First generation oncolytic viruses such as HSV-1 strain 17 mutant 1716 show significant therapeutic potential in tumour and gene therapy. Overcoming the existing technical difficulties by enabling rapid generation and screening of second generation oncolytic viruses of this kind provides a significant improvement in the development of novel pharmaceutical compositions, vaccines and medicaments for the treatment of cancer and disease.

HSV 1716 is described in EP 0571410 and WO 92/13943 and has been deposited on 28 January 1992 at the European Collection of Animal Cell Cultures, Vaccine Research and Production Laboratories, Public Health Laboratory Services, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number V92012803 in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (herein referred to as the 'Budapest Treaty').

Summary of the Invention

The inventors have provided a generic plasmid vector designated RL1.dIRES-GFP. RL1.dIRES-GFP provides a platform for generating a plurality of 'shuttle vectors' which can exploit the process of homologous recombination to transfer a nucleotide sequence of interest (downstream of a selected promoter) into the disabling RL1 locus of HSV-1, generating easily identifiable, oncolytic, ICP34.5 null HSV-1 mutants expressing the products of the nucleotide sequence of interest, e.g. an RNA transcript or a polypeptide, and GFP. RL1.dIRES-GFP thus provides for ease of generation and purification of ICP34.5 null HSV.

RL1.dIRES-GFP is a useful vector for making second-generation oncolytic viruses having enhanced cytotoxic potential and which may express the product(s) of selected gene(s) to enhance the oncolytic and/or therapeutic effect of the administered virus.

The RL1.dIRES-GFP plasmid incorporates a multi-cloning sequence (MCS), upstream of an internal ribosome entry site (IRES), the GFP gene and SV40 polyadenylation sequences flanked by HSV-1 RL1 sequences. Incorporation of the encephalomyocarditis virus IRES (EMCV IRES) permits translation of two open reading frames from a single transcribed mRNA.

Following generation of a specific shuttle vector by cloning of the nucleotide sequence of interest (and the selected promoter) into RL1.dIRES-GFP, recombinant HSV-1 expressing the desired nucleic acid transcript or protein, can be generated and purified within 2 weeks. This compares with 2-3 months using prior art protocols.

In the ICP34.5 null HSV generated using the RL1.dIRES-GFP plasmid provided by the inventors transcription of both the nucleotide sequence of interest and GFP as a single transcript is controlled by the same promoter upstream of the nucleotide sequence of interest, the transcribed IRES directing cap-independent translation of GFP. The generated ICP34.5 null HSV are non-neurovirulent. By modifying the RL1.dIRES-GFP plasmid to incorporate appropriate flanking sequences surrounding the cassette other gene-specific HSV null mutants expressing GFP can be generated.

RL1.dIRES-GFP is promoterless, thus enabling a promoter of choice to be incorporated in the homologously recombined shuttle vector for controlling expression of the nucleotide sequence of interest from the inserted cassette.

Plasmid RL1.dIRES-GFP or modified plasmid shuttle vectors thereof further comprising nucleotide sequence encoding a nucleic acid transcript or polypeptide of interest may be provided in isolated or purified form.

By using the plasmid RL1.dIRES-GFP to generate a shuttle vector, designated RL1.dCMV-NTR-GFP, containing the E.coli nitroreductase gene downstream of a CMV IE promoter, both inserted at the MCS, the inventors have further provided a novel second generation oncolytic mutant HSV. The genome of this mutant HSV comprises the heterologous (i.e. non-HSV) E.coli nitroreductase protein coding sequence inserted at one or each ICP34.5 locus, disrupting the ICP34.5 protein coding sequence such that the ICP34.5 gene is non-functional and cannot express a functional ICP34.5 gene product. The generated HSV is capable of expressing the E.coli nitroreductase gene product under control of the inserted promoter. This virus thus has the oncolytic activity of HSV strain 1716 and can be used in gene directed enzyme-prodrug therapy and has shown significantly enhanced tumour cell killing in vitro and in vivo when used with the prodrug CB1954. The mutant virus is designated HSV1716/CMV-NTR/GFP.

As the plasmid RL1.dIRES-GFP is designed for tandem expression of a sequence of interest and the marker gene encoding green fluorescent protein (GFP). The sequence of interest is cloned into RL1.dIRES-GFP along with its promoter (e.g. CMV) such that the promoter drives transcription of an mRNA for the sequence of interest along with the IRES-GFP. Translation results in

expression of the GFP from the internal ribosomal entry site and the gene of interest and promoter must be cloned into RL1.dIRES-GFP in the correct orientation to achieve this. There are a number of instances where this tandem expression arrangement may be unsuitable and a variation of the cassette design is favourable.

One example is the expression of siRNAs as short hairpin RNAs using RNA polIII promoters such as H1 or U6. These promoters are unable to drive the additional tandem expression of the IRES-GFP as the RNApolIII expression cassette is designed only to produce short transcripts. Additionally, sequences of interest derived from genomic DNA with strong mRNA shut-off signals in their 3' untranslated regions may not support IRES-GFP expression.

Thus in some cases a cassette may be provided in which the sequence of interest and marker are expressed separately from independent promoters.

One variant contains a cassette in which the ribosome binding site of plasmid RL1.dIRES-GFP is replaced with a regulatory nucleotide sequence, preferably a strong, constitutive promoter such as the Phosphoglycerokinase (PGK) promoter. The marker is thereby expressed under the control of this (the 'first') regulatory sequence. The nucleotide sequence of interest (e.g. antisense or siRNA) is expressed under the control of a second regulatory sequence upstream (5') of the nucleotide sequence of interest, e.g. the CMV promoter. This vector variant is particularly suitable for expression of siRNA where a weak promoter may be used for expression of the

siRNA molecule. It may also be useful where the nucleotide sequence of interest has a strong transcription and/or translation termination signal which may make it difficult to transcribe and/or translate a single bi- or poly-cistronic transcript encoding the sequence of interest and marker sequence. In this arrangement the transformed virus containing the cassette integrated in the viral genome produces two separate transcripts under the control of the first and second promoters.

At its most general the present invention relates to: (i) a nucleic acid vector for delivery of a nucleic acid cassette to an insertion site in a selected viral genome; (ii) an herpes simplex virus wherein the herpes simplex virus genome comprises a nucleotide sequence of interest.

The present invention further comprises novel HSV mutants and viral vectors which may be generated using nucleic acid vectors of the present invention, or vectors derived therefrom, and methods for the generation of such vectors and HSV mutants.

According to one aspect of the present invention there is provided a nucleic acid vector comprising, consisting or consisting essentially of:
first and second nucleotide sequences corresponding to nucleotide sequences flanking a predetermined insertion site in the genome of a selected herpes simplex virus (HSV); and a cassette located between said first and second nucleotide sequences comprising nucleic acid encoding:

(a) one or a plurality of insertion sites; and
(b) a ribosome binding site; and
(c) a marker.

Preferably the nucleic acid encoding the one or plurality of insertion sites is/are arranged upstream (5′) of the ribosome binding site and the nucleic acid encoding the ribosome binding site is arranged upstream (5′) of the marker.

According to another aspect of the present invention there is provided a nucleic acid vector comprising, consisting or consisting essentially of:
first and second nucleotide sequences corresponding to nucleotide sequences flanking a predetermined insertion site in the genome of a selected herpes simplex virus (HSV); and a nucleic acid cassette located between said first and second nucleotide sequences comprising:

(a) a third nucleotide sequence being of interest; and nucleic acid encoding:
(b) a ribosome binding site; and
(c) a marker.

Preferably the nucleotide sequence of interest is arranged upstream (5′) of the ribosome binding site and the ribosome binding site is arranged upstream (5′) of the marker.

According to another aspect of the present invention there is provided an herpes simplex virus wherein the herpes simplex virus (HSV) comprises a nucleic acid cassette integrated in the HSV genome, the cassette

comprising, consisting or consisting essentially of nucleic acid encoding:

(a) one or a plurality of insertion sites; and

(b) a ribosome binding site, and a

(c) marker.

Preferably, the nucleic acid encoding the one or plurality of insertion sites is/are arranged upstream (5′) of the ribosome binding site and the nucleic acid encoding the ribosome binding site is arranged upstream (5′) of the marker.

According to another aspect of the present invention there is provided an herpes simplex virus wherein the herpes simplex virus (HSV) comprises a nucleic acid cassette integrated in the HSV genome, the cassette comprising, consisting or consisting essentially of nucleic acid encoding:

(a) a nucleotide sequence of interest; and nucleic acid encoding:

(b) a ribosome binding site; and

(c) a marker.

Preferably, the nucleotide sequence of interest is arranged upstream (5′) of the ribosome binding site and the ribosome binding site is arranged upstream (5′) of the marker.

In various aspects of the present invention the ribosome binding site may comprise an internal ribosome entry site (IRES). A transcription product of the cassette may be a bi- or poly- cistronic transcript comprising a first

cistron encoded by the nucleotide sequence of interest and a second cistron encoding the marker nucleic acid wherein the ribosome binding site is located between said first and second cistrons.

According to another aspect of the present invention there is provided a nucleic acid vector comprising, consisting or consisting essentially of:
first and second nucleotide sequences corresponding to nucleotide sequences flanking a predetermined insertion site in the genome of a selected herpes simplex virus (HSV); and a cassette located between said first and second nucleotide sequences comprising nucleic acid encoding:

(a) one or a plurality of insertion sites; and
(b) a first regulatory nucleotide sequence; and
(c) a marker.

Preferably the nucleic acid encoding the one or plurality of insertion sites is/are arranged upstream (5′) of the regulatory sequence and the nucleic acid encoding the regulatory sequence is arranged upstream (5′) of the marker.

According to another aspect of the present invention there is provided a nucleic acid vector comprising, consisting or consisting essentially of:
first and second nucleotide sequences corresponding to nucleotide sequences flanking a predetermined insertion site in the genome of a selected herpes simplex virus (HSV); and a nucleic acid cassette located between said first and second nucleotide sequences comprising:

(a) a third nucleotide sequence being of interest; and nucleic acid encoding:
(b) a first regulatory nucleotide sequence; and
(c) a marker.

Preferably the nucleotide sequence of interest is arranged upstream (5′) of the regulatory sequence and the regulatory sequence is arranged upstream (5′) of the marker.

According to another aspect of the present invention there is provided an herpes simplex virus wherein the herpes simplex virus (HSV) comprises a nucleic acid cassette integrated in the HSV genome, the cassette comprising, consisting or consisting essentially of nucleic acid encoding:
(a) one or a plurality of insertion sites; and
(b) a first regulatory nucleotide sequence; and
(c) a marker.

Preferably the nucleic acid encoding the one or plurality of insertion sites is/are arranged upstream (5′) of the regulatory sequence and the nucleic acid encoding the regulatory sequence is arranged upstream (5′) of the marker.

According to another aspect of the present invention there is provided an herpes simplex virus wherein the herpes simplex virus (HSV) comprises a nucleic acid cassette integrated in the HSV genome, the cassette comprising, consisting or consisting essentially of nucleic acid encoding:

(a) a nucleotide sequence of interest; and nucleic acid encoding:
(b) a first regulatory nucleotide sequence; and
(c) a marker.

Preferably the nucleotide sequence of interest is arranged upstream (5′) of the regulatory sequence and the regulatory sequence is arranged upstream (5′) of the marker.

In the various aspects the encoded components of the cassette are preferably arranged in a predetermined order as described.

The first regulatory sequence may be operably linked to said marker and may comprise a constitutive or inducible promoter. The first regulatory sequence may thus have a role in regulating transcription of the marker.

The nucleotide sequence of interest may encode an heterologous polypeptide, which may be selected from the group consisting of: a bacterial polypeptide; a mammalian polypeptide; a human polypeptide or more particularly from the group consisting of: Sodium iodide symporter (NIS); Nitroreductase (NTR); E.coli NTR; Endothelial nitric oxide synthase (eNOS); Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF); a cytokine.

Alternatively, the nucleotide sequence of interest may encode a selected antisense nucleic acid or siRNA.

The cassette may further comprise a regulatory nucleotide sequence, located upstream (5′) of the nucleotide sequence of interest or insertion site(s), which has a role in regulating transcription of the nucleotide sequence of interest.

The cassette may comprise a plurality of insertion sites, each insertion site preferably formed by nucleic acid encoding a specific restriction endonuclease site ('restriction site'). Together the restriction sites may form a multiple cloning site (MCS) comprising a series of overlapping or distinct restriction sites, preferably a series of distinct restriction sites comprising one or more of the ClaI, BglII, NruI, XhoI restriction sites.

Accordingly, vectors and herpes simplex viruses according to the present invention may further comprise one or a plurality of insertion sites, more preferably restriction endonuclease sites encoded by nucleic acid of the cassette. Each insertion site may be formed by nucleic acid encoding a restriction endonuclease site. The insertion sites may comprise one or more of the ClaI, BglII, NruI and XhoI restriction endonuclease sites.

The predetermined insertion site is preferably in the RL1 locus of the genome of the selected herpes simplex virus.

The first and second nucleotide sequences may each comprise sequence corresponding to:

(a) nucleotide sequences in the RL terminal or internal repeat region of the genome of the selected HSV;

(b) nucleotide sequences in the RL1 locus of the genome of the selected HSV;

(c) nucleotide sequences flanking a predetermined insertion site formed in, or comprising all or a part of, the ICP34.5 protein coding sequence of the genome of a selected herpes simplex virus;

In other arrangements said first and second nucleotide sequences may comprise contiguous portions of nucleotide sequence of the ICP34.5 gene of a herpes simplex virus. Or said first and second nucleotide sequences may comprise contiguous portions of nucleotide sequence encoding the ICP34.5 gene product of a herpes simplex virus.

The first and second nucleotide sequences may have at least 60% sequence identity to their corresponding sequence in the viral genome. More preferably said identity may be one of at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%. Identity of sequences is determined across the entire length of a given nucleotide sequence. Where sequences are of different length, sequence identity of the shorter sequence is determined over the entire length of the longer sequence.

In certain arrangements the first and second nucleotide sequences may hybridise to their corresponding nucleotide sequence in the HSV genome, or its complement, under high or very high stringency conditions.

The marker may be a defined nucleotide sequence encoding a polypeptide. In one arrangement the marker may comprise the Green Fluorescent Protein (GFP) protein coding sequence or the enhanced Green Fluorescent Protein (EGFP) protein coding sequence.

In another arrangement the marker may comprise a defined nucleotide sequence detectable by hybridisation under high or very high stringency conditions with a corresponding labelled nucleic acid probe.

The cassette may further comprise a nucleic acid encoding a polyadenylation sequence located downstream (3′) of the nucleic acid encoding the marker. The polyadenylation sequence may comprise the Simian Virus 40 (SV40) polyadenylation sequence.

Preferably, mutant HSV according to the present invention are generated by site directed insertion of the cassette into the viral genome, more preferably by homologous recombination.

The vector may further comprise nucleic acid encoding a second selectable marker, e.g. a marker conferring antibiotic resistance.

The vector is preferably a DNA vector, particularly a dsDNA vector and may be an expression vector.

In one preferred arrangement, the vector is plasmid RL1.dIRES-GFP deposited in the name of Crusade Laboratories Limited having an address at Department of

Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 03 September 2003 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03090303 in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (herein referred to as the 'Budapest Treaty').

The selected herpes simplex virus may be an HSV-1 or HSV-2. Thus, mutant HSV according to the present invention may be derived from a strain of either HSV-1 or HSV-2. More preferably the selected HSV may be one of HSV-1 strains 17 or F or HSV-2 strain HG52. Thus, herpes simplex viruses of the invention may be mutants of these preferred 'parental' viruses.

In herpes simplex viruses according to the present invention the cassette may be integrated in the HSV genome so as to disrupt a protein coding sequence in the HSV genome resulting in inactivation of the respective gene product.

Herpes simplex virus of the invention may be gene specific null mutants. They may be ICP34.5 null mutants.

In some aspects of the present invention the herpes simplex virus may lack at least one expressible ICP34.5 gene. In other aspects of the present invention the generated herpes simplex virus may lack only one expressible ICP34.5 gene.

Herpes simplex virus according to the present invention may be non-neurovirulent.

Vectors and herpes simplex viruses according to the present invention may be used in gene therapy.

According to another aspect of the present invention there is provided a method of generating a herpes simplex virus which expresses a nucleotide sequence of interest, or polypeptide thereby encoded, comprising the step of culturing a selected herpes simplex virus with a vector according to the present invention, thereby integrating components (a), (b) and (c) of said vector at said predetermined insertion site in the genome of the selected herpes simplex virus.

Said integration at the predetermined insertion site is preferably a permanent integration.

According to another aspect of the present invention there is provided a method of generating a nucleic acid vector comprising the steps of:

i) providing a first nucleotide sequence comprising a predetermined second nucleotide sequence corresponding to a selected nucleotide sequence in the genome of a selected Herpes simplex virus; and

ii) inserting nucleotide sequence(s) in said second nucleotide sequence encoding:

a) one or a plurality of insertion sites and/or a nucleotide sequence of interest; and

b) a ribosome binding site or a regulatory nucleotide sequence; and

c) a marker.

Preferably the insertion site(s)/nucleotide sequence of interest is arranged upstream (5′) of the ribosome binding site/ regulatory nucleotide sequence and the ribosome binding site / regulatory nucleotide sequence is arranged upstream (5′) of the marker.

Said predetermined second nucleotide sequence may preferably correspond to a selected nucleotide sequence in the RL1 locus of the genome of the selected HSV.

The inserted nucleotide sequence(s) may separate the second nucleotide sequence into two vector flanking sequences, the inserted nucleotide sequences forming a cassette therebetween.

The second nucleotide sequence may correspond to a:

(a) nucleotide sequence in the RL terminal or internal repeat region of the genome of the selected herpes simplex virus;

(b) nucleotide sequence in the RL1 locus of the genome of the selected herpes simplex virus;

(c) a nucleotide sequence formed in, or comprising all or a part of, the ICP34.5 protein coding sequence of the genome of a selected herpes simplex virus.

The second nucleotide sequence may comprise a contiguous portion of nucleotide sequence of the ICP34.5 gene of a herpes simplex virus. Alternatively the second nucleotide sequence may comprise a contiguous portion of

nucleotide sequence encoding the ICP34.5 gene product of a herpes simplex virus.

The second nucleotide sequence may have at least 60% sequence identity to the corresponding sequence in the viral genome. More preferably said identity may be one of at least 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%. Identity of sequences is determined across the entire length of a given nucleotide sequence. Where sequences are of different length, sequence identity of the shorter sequence is determined over the entire length of the longer sequence.

Said second nucleotide sequence may hybridise to the corresponding nucleotide sequence in the viral genome, or its complement, under high or very high stringency conditions.

According to another aspect of the present invention there is provided a method of generating a mutant herpes simplex virus (HSV) comprising inserting a nucleic acid cassette comprising nucleotide sequence(s) encoding:

a) one or a plurality of insertion sites and/or a nucleotide sequence of interest; and

b) a ribosome binding site or a regulatory nucleotide sequence; and

d) a marker

into the genome of a selected HSV.

Preferably the insertion site(s)/nucleotide sequence of interest is arranged upstream (5′) of the ribosome binding site/ regulatory nucleotide sequence and the

ribosome binding site/ regulatory nucleotide sequence is arranged upstream (5′) of the marker.

The site of insertion may preferably be a predetermined insertion site in the RL1 locus of the selected HSV.

The method of generating a mutant herpes simplex virus may comprise the steps of:

i) providing a vector according to the present invention;

ii) where the vector is a plasmid, linearising the vector; and

iii) co-transfecting a cell culture with the linearised vector and genomic DNA from said HSV.

The co-transfection may be carried out under conditions effective for homologous recombination of said cassette into an insertion site in the viral genome.

The method of generating a mutant herpes simplex virus may further comprise one or more of the steps of:

1) screening said co-transfected cell culture to detect mutant HSV expressing said marker; and/or

2) isolating said mutant HSV; and/or

3) screening said mutant HSV for expression of the nucleotide sequence of interest or the RNA or polypeptide thereby encoded; and/or

4) screening said mutant HSV for lack of an active gene product; and/or

5) testing the oncolytic ability of said mutant HSV to kill tumour cells in vitro.

In the method of generating a mutant herpes simplex virus the nucleotide sequence of interest may be heterologous to the selected herpes simplex virus. It may encode an heterologous polypeptide which may be selected from the group consisting of: a bacterial polypeptide; a mammalian polypeptide; a human polypeptide or from the group consisting of: Sodium iodide symporter (NIS); Nitroreductase (NTR); E.coli NTR; Endothelial nitric oxide synthase (eNOS); Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF); a cytokine.

Alternatively the nucleotide sequence of interest may encode a selected antisense nucleic acid.

In another aspect of the present invention there is provided a mutant herpes simplex virus generated by the method of generation disclosed herein. Herpes simplex viruses generated by such methods may be gene specific null mutants, e.g. ICP34.5 null mutants.

The present invention may also include the following aspects and features which may be provided in combination with any of the other aspects and features described.

In aspects of the present invention the nucleotide sequence of interest contained in the cassette may encode a polypeptide of interest, or fragment thereof, or comprise selected antisense DNA, that is DNA corresponding to at least one gene component (e.g. regulatory sequence 5' UTR, 3'UTR or protein coding sequence) or fragment of a gene component, which is inserted in the cassette in an orientation such that upon

transcription an antisense RNA is obtained. Thus the expressed product of the nucleotide sequence of interest may ultimately be a polypeptide, complete or truncated (e.g. a polypeptide fragment), or an antisense nucleic acid, preferably RNA.

By antisense nucleic acid is meant a nucleic acid having substantial sequence identity to the nucleic acid formed by the sequence of complementary bases to the single strand of a target nucleic acid. Thus, the antisense nucleic acid is useful in binding the target nucleic acid and may be used as an inhibitor to prevent or disrupt the normal activity, folding or binding of the target nucleic acid. The substantial sequence identity is preferably at least 50% sequence identity, more preferably at least 60, 70, 75, 80, 85, 90, 92, 94, 95, 96, 97, 98, 99 or 100% identity. Identity of sequences is determined across the entire length of a given nucleotide sequence. Where sequences are of different length, sequence identity of the shorter sequence is determined over the entire length of the longer sequence.

Where the nucleotide sequence of interest encodes a polypeptide of interest the polypeptide may be any selected polypeptide. Preferably, the polypeptide of interest is an heterologous or exogenous polypeptide (i.e. a non-HSV originating polypeptide), preferably a bacterial polypeptide, alternatively a mammalian polypeptide or a human polypeptide. The heterologous polypeptide may be useful in gene directed enzyme-prodrug targeting techniques for tissue specific delivery of active pharmaceutical agents. For example, the

polypeptide of interest may be the Sodium iodide symporter (NIS), Nitroreductase (NTR), preferably E.coli NTR, Endothelial nitric oxide synthase (eNOS), Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) or a cytokine.

The cassette preferably further comprises at least one regulatory nucleotide sequence such as one or more selected promoter or enhancer elements known to the person skilled in the art, e.g. the CytoMegalovirus (CMV) promoter or Phosphoglycerokinase (PGK) promoter . A regulatory nucleotide sequence may be located upstream (i.e. 5') of the nucleotide sequence of interest and have a role in controlling and regulating transcription of the nucleotide sequence of interest and hence expression of the resulting transcript or polypeptide. In certain arrangements another regulatory sequence may be located upstream (5') of the marker sequence and downstream (3') of the nucleotide sequence of interest and have a role in controlling and regulating transcription of the marker and hence expression of the resulting transcript or polypeptide. In some arrangements this latter regulatory sequence may take the place of the ribosome binding site.

The components of the cassette are preferably arranged in a predetermined order. In certain asepcts, the nucleotide sequence of interest is arranged upstream (i.e. 5') of the ribosome binding site and the ribosome binding site is arranged upstream (i.e. 5') of the marker. Thus during transcription a single transcript may be produced from the cassette comprising a first cistron comprising the nucleotide sequence of interest and a second cistron

encoding the marker wherein the ribosome binding site is located between the cistrons.

A suitable ribosome binding site may comprise a ribosome entry site permitting entry of a ribosome to the transcribed mRNA encoded by the nucleic acid of the cassette such that the ribosome binds to the translation start signal. Preferably, the ribosome entry site is an internal ribosome entry site (IRES), more preferably an encephalomyocarditis virus IRES, permitting cap-independent initiation of translation. The IRES thus enables translation of a coding sequence located internally of a bi- or poly- cistronic mRNA, i.e. of a cistron located downstream of an adjacent cistron on a single transcript.

Preferably the marker is a defined nucleotide sequence coding for a polypeptide which can be expressed in a cell line (e.g. BHK cells) infected with mutant herpes simplex virus into which the cassette has been recombined. One function of the marker is to enable identification of virus plaques containing mutant virus transformed with the cassette.

Alternatively, the marker may comprise a defined nucleotide sequence which can be detected by hybridisation under high stringency conditions with a corresponding labelled nucleic acid probe, e.g. using a fluorescent- or radio-label.

The marker is preferably a detectable marker, more preferably an expressible marker polypeptide or protein

comprising at least the coding sequence for the selected polypeptide or protein. The nucleic acid encoding the marker may further comprise regulatory sequence upstream and/or downstream of the coding sequence having a role in control of transcription of the marker mRNA. Preferred markers include the Green Fluorescent Protein (GFP) protein coding sequence or gene, preferably the enhanced Green Fluorescent Protein (EGFP) protein coding sequence or gene.

In another preferred arrangement, the cassette further comprises a polyadenylation sequence ('polyA sequence'). Preferably the polyA sequence comprises the Simian Virus 40 (SV40) polyA sequence. The preferred location of the polyA sequence within the cassette is immediately downstream (i.e. 3') of the marker.

The first and second nucleotide sequences preferably comprise nucleotide sequences having identity to regions of the genome surrounding the insertion site in the selected herpes simplex virus strain (the 'viral insertion site'). These sequences enable the cassette to be incorporated at the viral insertion site by homologous recombination between the first and second nucleotide sequences and their respective corresponding sequences in the viral genome.

Thus the first and second nucleotide sequences are flanking sequences for homologous recombination with corresponding sequences of a selected viral genome, such homologous recombination resulting in insertion of the cassette at the viral insertion site.

Preferably, the first and second nucleotide sequences correspond to nucleotide sequences flanking an insertion site in the RL1 locus of the HSV genome, more preferably in the ICP34.5 protein coding sequence of the HSV genome.

The first and second nucleotide sequences may each be at least 50bp in length, more preferably at least 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000bp in length. Each of the first and second nucleotide sequences may have at least 50% sequence identity to their corresponding sequence in the viral genome, more preferably at least 60%, 70%, 75%, 80%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% 99% or 100% identity. Identity of sequences is determined across the entire length of a given nucleotide sequence. Where sequences are of different length, sequence identity of the shorter sequence is determined over the entire length of the longer sequence.

The first and second nucleotide sequences may be characterised by the ability of one strand of a given sequence to hybridise with the corresponding single-stranded complement of the HSV genome under varying hybridisation stringency conditions. Suitably, the first and second nucleotide sequences will hybridise with their corresponding complement under very low, low or intermediate stringency conditions, more preferably at high or very high stringency conditions.

The nucleotide sequence of interest which forms part of the inserted cassette may encode a full length transcript or polypeptide (i.e. comprise the complete protein coding sequence). Alternatively, the nucleotide sequence of interest may comprise one or more fragments of the full length sequence respectively coding for a fragment of the full length transcript or a truncated polypeptide or antigenic peptide respectively. A fragment may comprise a nucleotide sequence encoding at least 10% of the corresponding full length sequence, more preferably the fragment comprises at least 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98 or 99% of the corresponding full length sequence. Preferably, the fragment comprises at least, i.e. has a minimum length of, 20 nucleotides, more preferably at least 30, 40, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000 nucleotides. The fragment may have a maximum length, i.e. be no longer than, 20 nucleotides, more preferably no longer than 30, 40, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or 4000. The fragment length may be anywhere between said minimum and maximum length.

The viral insertion site is the position between the genomic nucleotide sequences corresponding to the first

and second nucleotide sequences of the vector (the 'genomic' and 'vector flanking sequences' respectively) at which homologous recombination will occur and may be predetermined by selection of the vector flanking sequences. Where the genomic flanking sequences are immediately adjacent, the insertion site is the position between the peripheral and immediately adjacent bases of the two genomic flanking sequences, such that insertion of the cassette separates the genomic flanking sequences. Where the genomic flanking sequences are separated by one or a plurality of bases in the viral genome, the insertion site is formed by said one or a plurality of bases which are excised from the genome by the homologous recombination event.

The position of the viral insertion site may be accurately selected by careful selection and construction of the vector flanking sequences. Accordingly, the vector may be constructed such that homologous insertion of the cassette results in disruption of a chosen protein coding sequence and inactivation of the respective gene product or such that the cassette is inserted at a non-protein coding region of the viral genome. The complete genome sequences of several herpes simplex virus strains have been reported and are publicly available. The complete genome sequence for HSV-1 strain 17syn+ was reported by Dolan et al[3] (incorporated herein by reference) and the complete genome sequence of HSV-2 strain HG52 was reported by Dolan et al[4] (incorporated herein by reference) and is available from the EMBL database under accession code Z86099. Using this information, the vector of the present invention may preferably be designed for

use in generating mutant HSV-1 (e.g. in strain 17 or F) or mutant HSV-2 (e.g. in strain HG52).

Preferably the first and second nucleotide sequences (vector flanking sequences) each comprise sequence corresponding to the RL terminal repeat region of the genome of the selected HSV (e.g. HSV-1 strains 17 or F or HSV-2 strain HG52). More preferably, vector flanking sequences comprise, consist or consist essentially of nucleotide sequences of the RL repeat region which flank the ICP34.5 protein coding sequence. In flanking the ICP34.5 coding sequence, one or both of the selected sequences may, in the corresponding HSV genome, overlap, i.e. extend into, the ICP34.5 protein coding sequence or one or both sequences may be selected so as to not overlap the ICP34.5 protein coding sequence or so as to be within it entirely. In a similar manner, the selected sequences may be chosen to overlap completely or partially other important encoded signals, e.g. transcription initiation site, polyadenylation site, defined promoters or enhancers. In this preferred arrangement the insertion site may thus comprise all or a part of the ICP34.5 protein coding sequence and/or be such that the inserted cassette disrupts the ICP34.5 protein coding sequence.

Thus, vectors according to the present invention comprising first and second nucleotide sequences corresponding to regions of the RL repeat region flanking and/or overlapping or being entirely within the ICP34.5 protein coding sequence may be used in the generation of ICP34.5 null mutants. In such mutants all or a portion of

the ICP34.5 protein coding sequence may be excised and replaced during the homologous recombination event such that both copies of the ICP34.5 coding sequence are disrupted. Alternatively, the recombination may result in an insertion of nucleic acid within the ICP34.5 protein coding sequence thereby disrupting that sequence. In that case, successfully transformed virus are thus mutants incapable of generating the ICP34.5 active gene product from at least one copy, and preferably from both copies, of the ICP34.5 gene.

Where all copies of the ICP34.5 gene present in the herpes simplex virus genome (two copies are normally present) are disrupted such that the herpes simplex virus is incapable of producing a functional ICP34.5 gene product, the virus is considered to be an ICP34.5 null mutant.

Preferably, each component of the cassette is positioned substantially adjacent the neighbouring component such that a single bicistronic transcript comprising or consisting essentially of the mRNA encoding the nucleotide sequence of interest, ribosome binding site and marker is obtainable.

Preferably, the vector further comprises, consists, or consists essentially of a nucleic acid encoding a selectable marker such as a polypeptide or protein conferring antibiotic resistance e.g. kanamycin resistance or ampicillin resistance.

A vector of the present invention preferably comprises a DNA vector, particularly a dsDNA vector. The vector may be provided as a linear or circular (plasmid) DNA vector. The vector preferably contains nucleotide sequences, e.g. restriction endonuclease site(s), permitting transition between the two forms by use of DNA ligation and restriction materials (e.g. enzymes) and techniques known to the person skilled in the art. To achieve homologous recombination with a selected HSV strain, the vector is preferably provided in linear form.

In one preferred arrangement, the vector is plasmid RL1.dIRES-GFP deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 03 September 2003 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03090303 in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (herein referred to as the 'Budapest Treaty').

In another preferred arrangement, the vector is a variant of plasmid RL1.dIRES-GFP.

Variant vectors may incorporate a regulatory nucleotide sequence, e.g. promoter, in place of the ribosome binding site of RL1.dIRES-GFP to control transcription of the nucleic acid encoding the marker.

Vectors according to the present invention are preferably constructed for use in generating engineered HSV-1 or HSV-2 by insertion of a nucleic acid cassette, which insertion may be through a mechanism of homologous recombination between nucleotide sequences flanking the cassette and corresponding sequences in the selected herpes simplex virus genome. The cassette may thereby be permanently integrated at a selected site of the viral genome.

Thus, vectors according to the present invention may comprise and have use as:

i) gene delivery (gene therapy) vectors for delivery of a selected protein coding sequence or antisense nucleic acid to a specific locus of the HSV genome; and/or

ii) expression vectors for expression of the delivered protein coding sequence or antisense nucleic acid of i) from the HSV genome under the control of a selected regulatory element; and/or

iii) vectors for the generation of HSV gene-specific mutants (optionally null mutants) wherein the cassette is inserted at a selected genomic location to disrupt the protein coding sequence of a selected HSV gene such that the gene product is inactive in the resultant mutant virus.

Vectors according to the present invention may be used in the manufacture of engineered gene specific HSV null mutants, i.e. HSV mutants incapable of expressing an active gene product of a selected gene. Alternatively they may be used in the manufacture of engineered viruses

which express a selected protein from only one gene copy the other gene copy being disrupted or modified such that it cannot express a functional gene product. Such vectors may also be used in the manufacture of a medicament, preferably comprising said gene specific HSV (null) mutant, for use in treating tumours, preferably by the oncolytic treatment of the tumour. Preferably, such tumours may be primary or secondary (metastatic) tumours originating either in the central or peripheral nervous system, e.g. glioma, medulloblastoma, meningioma, neurofibroma, ependymoma, Schwannoma, neurofibrosarcoma, astrocytoma and oligodendroglioma, or originating in non-nervous system tissue e.g. melanoma, mesothelioma, lymphoma, hepatoma, epidermoid carcinoma, prostate carcinoma, breast cancer cells, lung cancer cells or colon cancer cells. HSV mutants generated using vectors of the present invention may be used to treat metastatic tumours of the central or peripheral nervous system which originated in a non-nervous system tissue.

Vectors according to the present invention may also be used in the manufacture of engineered HSV mutants wherein the genome of the mutant HSV comprises an exogenous or heterologous gene which may have been inserted in the HSV genome by homologous recombination of the cassette. Preferably, the exogenous/heterologous gene is expressed in the mutant HSV, which expression may be regulated by a regulatory element, e.g. promoter, forming part of the inserted cassette. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

Vectors according to the present invention may also be used in the manufacture of an engineered HSV mutant wherein the genome of the mutant HSV comprises an exogenous/ heterologous gene (i.e. a non-HSV originating gene) which may have been inserted in a protein coding sequence of the HSV genome by homologous recombination of the cassette such that the mutant HSV is incapable of expressing the active gene encoded by said protein coding sequence and wherein the exogenous/ heterologous gene product is expressed under the control of a regulatory element. Preferably, the regulatory element forms part of the cassette. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

Vectors according to the present invention may also be used in the manufacture of engineered HSV mutants wherein the genome of the mutant HSV comprises a nucleotide sequence which may have been inserted in the HSV genome by homologous recombination of the cassette such that the nucleotide sequence is arranged to be transcribed from the HSV genome under the control of a regulatory element e.g. promoter, preferably a regulatory element forming part of the inserted cassette, to produce an antisense transcript. Preferably the antisense nucleotide sequence is an exogenous/ heterologous (i.e. non-HSV originating) sequence. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

Vectors according to the present invention may also be used in the manufacture of an engineered HSV mutant wherein the genome of the mutant HSV comprises a nucleotide sequence which may have been inserted in a protein coding sequence of the HSV genome by homologous recombination of the cassette such that the mutant HSV is incapable of expressing the active gene encoded by said protein coding sequence and wherein the inserted nucleotide sequence is expressed under the control of a regulatory element to produce an antisense transcript. Preferably, the regulatory element forms part of the cassette. Such vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

In another aspect of the present invention there is provided a method of lysing or killing tumour cells in vitro or in vivo comprising the step of administering mutant HSV, having a mutation in each ICP34.5 protein coding sequence and generated by a method according to an aspect of the present invention, to tumour cells.

In another aspect of the present invention there is provided a method of treating a tumour comprising administering to a subject mutant HSV having a mutation in each ICP34.5 protein coding sequence and generated by a method according to an aspect of the present invention.

In another aspect of the present invention there is provided a medicament, pharmaceutical composition or

vaccine comprising a mutant HSV generated by a method according to an aspect of the present invention. The medicament, pharmaceutical composition or vaccine is preferably for use in the oncolytic treatment of tumours and may further comprise a pharmaceutically acceptable carrier, adjuvant or diluent.

In another aspect of the present invention there is provided a kit of parts comprising a first container having a quantity of a vector according to an aspect of the present invention and a second container comprising a quantity of HSV genomic DNA.

In another aspect of the present invention there is provided a mutant HSV generated using the vector of, or vectors derived from, an aspect of the present invention. Preferably, the mutant is a gene specific null mutant, more preferably an HSV ICP34.5 null mutant, wherein the HSV genome comprises an inserted nucleotide sequence of interest encoding a selected antisense RNA or an heterologous polypeptide. Preferably the nucleotide sequence of interest has been inserted in each RL region of the HSV genome, more preferably at both of the ICP34.5 loci, still more preferably the inserted heterologous nucleic acid disrupts the ICP34.5 protein coding sequence such that both ICP34.5 genes are non-functional and the mutant HSV is incapable of expressing an active ICP34.5 gene product from the disrupted ICP34.5 protein coding sequences. Preferably, the inserted heterologous nucleotide sequence is non-endogenous to HSV and encodes a polypeptide of interest selected from the group comprising or consisting of Sodium iodide symporter

(NIS), Nitroreductase (NTR), preferably E.coli NTR, Endothelial nitric oxide synthase (eNOS), Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) or a cytokine.

The inserted nucleotide sequence of interest is preferably expressed or capable of expression under the control of an inserted regulatory element, preferably the CMV IE promoter. The mutant HSV genome preferably encodes the GFP gene product. More preferably the GFP coding sequence and nucleotide sequence of interest are arranged to be transcribed on a single bi- or poly-cistronic transcript such that expression of GFP is an indicator of HSV gene specific null mutants transfornmed with the nucleotide sequence of interest.

The nucleic acid cassette may be of any size, e.g. up to 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50Kbp in length.

In one preferred arrangement, the mutant HSV is HSV1716/CMV-NTR/GFP deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 05 November 2003 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03110501 in accordance with the provisions of the Budapest Treaty.

Medicaments comprising HSV mutants according to the present invention for use in oncotherapy and methods of treating tumours comprising administering to a patient in

need of treatment an effective amount of a mutant HSV according to an aspect of the present invention or a medicament comprising or derived from such HSV are also provided.

The use of such mutant HSV in the treatment of disease, including methods for the treatment of tumours/cancer, preferably by oncolysis is provided. Use of such mutant HSV in the manufacture of a medicament for use in these treatments is also provided.

Suitably, the administration of herpes simplex virus may comprise parenteral administration. Preferably administration of the herpes simplex virus is by injection, more preferably injection to the tumour which is to be treated.

Alternatively injections may be intravenous. Alternative administration routes may comprise oral or nasal administration.

Herpes simplex viruses according to the present invention may contain at least one copy of a nucleotide sequence of interest in each long repeat region (R_L), i.e. in the terminal and internal long repeat (TR_L and IR_L) regions. In a preferred arrangement each exogenous/heterologous sequence or cassette is located in an RL1 locus of the herpes simplex virus genome, more preferably in the DNA of the herpes simplex virus genome encoding the ICP34.5 gene or protein coding sequence. The herpes simplex virus thereby lacks neurovirulence.

The parent herpes simplex virus, from which a virus of the invention is derived may be of any kind, e.g. HSV-1 or HSV-2. In one preferred arrangement the herpes simplex virus is a variant of HSV-1 strain 17 and may be obtained by modification of the strain 17 genomic DNA. Suitable modifications include the insertion of the exogenous nucleotide sequence of interest or exogenous/ heterologous cassette comprising said sequence into the herpes simplex virus genomic DNA. The insertion may be performed by homologous recombination of the exogenous nucleic acid sequence into the genome of the selected herpes simplex virus.

Herpes simplex viruses of the present invention may be variants of a known 'parent' strain from which the herpes simplex virus of the invention has been derived. A particularly preferred parent strain is HSV-1 strain 17. Other parent strains may include HSV-1 strain F or HSV-2 strain HG52. A variant comprises an HSV in which the genome substantially resembles that of the parent, contains the nitroreductase nucleic acid sequence or cassette containing said sequence and may contain a limited number of other modifications, e.g. one, two or three other specific mutations, which may be introduced to disable the pathogenic properties of the herpes simplex virus, for example a mutation in the ribonucleotide reductase (RR) gene, the 65K trans inducing factor (TIF) and/or a small number of mutations resulting from natural variation, which may be incorporated naturally during replication and selection in vitro or in vivo. Otherwise the genome of the variant will be that of the parent strain.

In other aspects of the present invention vectors and/or herpes simplex viruses according to the present invention are provided for use in a method of medical treatment. Suitably they are provided for use in the treatment of disease. Preferably they are provided for use in the treatment of cancer. Suitably they may be provided for use in the oncolytic treatment of cancer/tumour of any kind. The use of vectors and/or herpes simplex viruses according to the present invention in the manufacture of a medicament for the treatment of cancer is also provided.

In another aspect of the present invention medicaments comprising herpes simplex virus mutants according to the present invention for use in oncotherapy and methods of treating tumours comprising administering to a patient in need of treatment an effective amount of a mutant HSV or a medicament comprising or derived from such HSV are also provided. Methods of lysing or killing tumour cells in vitro or in vivo comprising the step of administering to a patient in need of treatment an amount of an Herpes simplex virus according to the present invention are also provided.

A medicament, pharmaceutical composition or vaccine comprising a vector or Herpes simplex virus according to the present invention is also provided. The medicament, pharmaceutical composition or vaccine may further comprise a pharmaceutically acceptable carrier, adjuvant or diluent.

Herpes simplex viruses of the invention may be used in a method of medical treatment. This may involve treatment of diseases associated with or involving the proliferation of cells, or cancers or tumours of any kind. Treatment may involve the selective lysis of dividing cells. This may be oncolysis, i.e. lysis of tumour cells.

Tumours to be treated may be of any kind, may comprise cancers, neoplasms or neoplastic tissue and may be in any animal or human patient. Cancer/tumour types which may be treated may be primary or secondary (metastatic) tumours of any kind.

Treatable tumour types may include primary or secondary (metastatic) tumours originating either in the central or peripheral nervous system, e.g. glioma, medulloblastoma, meningioma, neurofibroma, ependymoma, Schwannoma, neurofibrosarcoma, astrocytoma and oligodendroglioma, or originating in non-nervous system tissue e.g. melanoma, mesothelioma, lymphoma, hepatoma, epidermoid carcinoma, prostate carcinoma, breast cancer cells, lung cancer cells or colon cancer cells. Treatable metastatic tumours may be those of the central or peripheral nervous system which originated in a non-nervous system tissue.

Herpes simplex viruses of the invention may be used in 'gene delivery' methods in vitro or in vivo. Non-neurovirulent herpes simplex viruses of the invention are expression vectors and may be used to infect selected cells or tissues in order to express the nucleotide

sequence of interest encoded by the herpes simplex virus genome.

Mutant HSV according to aspects of the present invention may be useful in the treatment of disease by gene directed enzyme-prodrug therapy and/or the treatment of disease, including tumours, by the use of antisense RNA or siRNA technology.

In one arrangement, cells may be taken from a patient, a donor or from any other source, infected with a herpes simplex virus of the invention, optionally screened for expression and/or function of the encoded nucleotide sequence of interest, and optionally returned/introduced to a patient's body, e.g. by injection.

Delivery of herpes simplex viruses of the invention to the selected cells may be performed using naked virus or by encapsulation of the virus in a carrier, e.g. nanoparticles, liposomes or other vesicles.

In vitro cultured cells, preferably human or mammalian cells, transformed with viruses of the present invention and preferably cells expressing the nucleotide sequence of interest as well as methods of transforming such cells in vitro with said viruses form further aspects of the present invention.

A nucleotide sequence of interest may be an exogenous/heterologous sequence, i.e. one not originating in the parent (wild-type) herpes simplex virus from which the herpes simplex virus of the invention is derived.

In this specification, a mutant herpes simplex virus is a non-wild type herpes simplex virus and may be a recombinant herpes simplex virus. Mutant herpes simplex viruses may comprise a genome containing modifications relative to the wild type. A modification may include at least one deletion, insertion, addition or substitution.

Medicaments and pharmaceutical compositions according to aspects of the present invention may be formulated for administration by a number of routes, including but not limited to, parenteral, intravenous, intramuscular, intratumoural, oral and nasal. The medicaments and compositions may be formulated in fluid or solid (e.g. tablet) form. Fluid formulations may be formulated for administration by injection to a selected region of the human or animal body.

In this specification, non-neurovirulence is defined by the ability to introduce a high titre of virus (approx 10^6 plaque forming units (pfu)) to an animal or patient[22, 23] without causing a lethal encephalitis such that the LD_{50} in animals, e.g. mice, or human patients is in the approximate range of $\geq 10^6$ pfu[21].

Where all copies of the ICP34.5 gene present in the herpes simplex virus genome (two copies are normally present) are disrupted such that the herpes simplex virus is incapable of producing a functional ICP34.5 gene product, the virus is considered to be an ICP34.5 null mutant.

A regulatory sequence (e.g. promoter) that is operably linked to a nucleotide sequence may be located adjacent to that sequence or in close proximity such that the regulatory sequence can effect and/or control expression of a product of the nucleotide sequence. The encoded product of the nucleotide sequence may therefore be expressible from that regulatory sequence.

Hybridisation stringency

In accordance with the present invention, nucleic acid sequences may be identified by using hybridization and washing conditions of appropriate stringency.

Complementary nucleic acid sequences will hybridise to one another through Watson-Crick binding interactions. Sequences which are not 100% complementary may also hybridise but the strength of the hybridisation usually decreases with the decrease in complementarity. The strength of hybridisation can therefore be used to distinguish the degree of complementarity of sequences capable of binding to each other.

The "stringency" of a hybridization reaction can be readily determined by a person skilled in the art.

The stringency of a given reaction may depend upon factors such as probe length, washing temperature, and salt concentration. Higher temperatures are generally required for proper annealing of long probes, while shorter probes may be annealed at lower temperatures. The higher the degree of desired complementarity between the

probe and hybridisable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so.

For example, hybridizations may be performed, according to the method of Sambrook et al., ("Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989) using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules is to calculate the melting temperature T_m (Sambrook et al., 1989):

$T_m = 81.5°C + 16.6Log [Na+] + 0.41(\% G+C) - 0.63 (\%$ formamide) $- 600/n$

where n is the number of bases in the oligonucleotide.

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in sequence complementarity.

Accordingly, nucleotide sequences can be categorised by an ability to hybridise to a target sequence under different hybridisation and washing stringency conditions which can be selected by using the above equation. The T_m may be used to provide an indicator of the strength of the hybridisation.

The concept of distinguishing sequences based on the stringency of the conditions is well understood by the person skilled in the art and may be readily applied.

Sequences exhibiting 95-100% sequence complementarity may be considered to hybridise under very high stringency conditions, sequences exhibiting 85-95% complementarity may be considered to hybridise under high stringency conditions, sequences exhibiting 70-85% complementarity may be considered to hybridise under intermediate stringency conditions, sequences exhibiting 60-70% complementarity may be considered to hybridise under low stringency conditions and sequences exhibiting 50-60%% complementarity may be considered to hybridise under very low stringency conditions.

The invention includes the combination of the aspects and preferred features described except where such a

combination is clearly impermissible or expressly avoided.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Brief Description of the Figures

Figure 1. Generation of plasmid RL1.dIRES-GFP from plasmids pNAT-IRES-GFP and RL1.del.

Figure 2. Agarose gel electrophoresis of *Hpa*I digested, CIP treated, RL1.del. RL1.del was digested with *Hpa*I. The digested DNA was then treated with Calf Intestinal Phosphatase (CIP) to prevent the vector re-annealing to itself in subsequent ligation reactions. A sample of the digested/CIP treated DNA was electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel. *Hpa*I linearises the vector at 8.6 Kbp.

Figure 3. Agarose gel electrophoresis of *Nsi*I/*Ssp*I digested pNAT-IRES-GFP (A) and purified/blunt-ended pCMV-NAT-IRES-GFP-PolyA (B). Four *Nsi*I/*Ssp*I digestions of pNAT-IRES-GFP were electrophoresed, beside a 1Kbp DNA Ladder (Promega) on a 1% agarose gel. The 5.4Kbp fragments (pCMV-NAT-IRES-GFP-PolyA) were purified from the gel. The purified DNA was blunt ended using Klenow

polymerase and a sample electrophoresed on an agarose gel to check its concentration.

Figure 4. Identification of RL1.del clones containing the pCMV-NAT-IRES-GFP-PolyA insert. Ligation reactions were set up with the purified, blunt ended pCMV-NAT-IRES-GFP-PolyA fragment and *Hpa*I digested, CIP treated RL1.del. Bacteria were transformed with samples from the ligation reactions and plated out onto LBA (Amp^r) plates. Colonies were picked and plasmid DNA was extracted and digested with *Afl*II. Digested samples were electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel. *Clones 5 and 8 contained the pCMV-NAT-IRES-GFP-PolyA insert as two fragments of the predicted size - 4.8Kbp and 9.2Kbp - were generated from *Afl*II digestion. Clones without inserts would not be digested with *Afl*II as there is no *Afl*II site in RL1.del.
N.B. Inserts could have been cloned in two orientations, both of which were acceptable.

Figure 5. Determination of the orientation of pCMV-NAT-IRES-GFP-PolyA in clone 5 (RL1.dCMV-NAT-GFPb). pCMV-NAT-IRES-GFP-PolyA (blunt ended) could have been cloned into the *Hpa*I site of RL1.del in two orientations. To determine the orientation of the insert in clone 5, the plasmid was digested with *Xho*I and the digested DNA electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel. If the insert had been cloned in the orientation shown in A, two fragments of 10.2Kbp and 3.8Kbp would be generated from *Xho*I digestion. If it had been cloned in the opposite orientation (B), two fragments of 12.4Kbp and 1.6Kbp would be generated. The

presence of two fragments of 10.2Kbp and 3.8Kbp in the gel confirmed that the insert had been cloned in the orientation shown in A.

*This *Xho*I site was present in the initial cloning vector (RL1.del), upstream of the *Hpa*I site into which pCMV-NAT-IRES-GFP-PolyA was cloned.

Figure 6. Removal of pCMV-NAT from clone 5 (A) and large scale plasmid preparation of RL1.dIRES-GFP (B). Four samples of clone 5 were digested with *Xho*I and electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel (A). The larger fragment of DNA generated from this digestion (10.2Kbp) was purified from the gel and ligated back together, at the *Xho*I sites, to form a single *Xho*I site in a new plasmid, designated RL1.dIRES-GFP. A large-scale plasmid preparation was grown up and the preparation checked by digesting with *Xho*I. 1µl and 4µl of the digested DNA was electrophoresed, beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel (B). The DNA should produce a single fragment of 10.2Kbp when digested with *Xho*I. The *Cla*I, *Bgl*II, *Nru*I and *Xho*I sites of RL1.dIRES-GFP are all unique.

*Clone 5 is the RL1.del plasmid into which has been cloned the 5.4Kbp pCMV-NAT-IRES-GFP-PolyA fragment from pNAT-IRES-GFP.

Figure 7. Generation, detection and purification of ICP34.5 null HSV-1 expresssing a gene product of interest.

Figure 8. Strategy used to clone pCMV-NTR from pPS949 into RL1.dIRES-GFP. (1) Digest pPS949 with *Bam*H1 and purify the 1.6Kbp pCMV-NTR fragment; (2) Digest RL1.dIRES-GFP with *Bgl*II and treat with Calf Intestinal Phosphatase (CIP); (3) Clone the pCMV-NTR fragment (*Bam*HI ends) into the *Bgl*II site of RL1.dIRES-GFP.
* The pPS949 plasmid was a kind gift from Professor Lawrence Young (University of Birmingham) and contains the *E.coli* nitroreductase (NTR) gene downstream of the CMV-IE promoter (pCMV) in pLNCX (Clontech).

Figure 9. Agarose gel electrophoresis of *Bam*HI digested pPS949 (A) and the purified pCMV-NTR fragment (B). Four samples of pPS949 were digested with *Bam*HI and electrophoresed, beside a 1Kbp DNA ladder (L) (New England Biolabs), on a 1% agarose gel. The 1.6Kbp fragments, consisting of the *E.coli* nitroreductase (NTR) gene downstream of the CMV IE promoter (pCMV), were purified from the gel and a sample of the purified DNA was electrophoresed on an agarose gel to check its concentration.

Figure 10. Agarose gel electrophoresis of *Bgl*II digested, CIP treated RL1.dIRES-GFP.RL1.dIRES.GFP was digested with *Bgl*II. The digested plasmid was then treated with Calf Intestinal Phosphatase (CIP) to prevent the vector re-annealing to itself in subsequent ligation reactions. A sample of the digested/CIP treated DNA was electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel to check its concentration. pCMV-NTR from pPS949 was subsequently cloned into this digested/CIP treated vector.

Figure 11. Determination of the orientation of pCMV-NTR in clone 4. pCMV-NTR (*Bam*HI ends) could have been cloned into the *Bgl*II site of RL1.dIRES-GFP in two orientations. To determine the orientation, clone 4 was digested with *Bgl*II and *Xho*I and the digested DNA electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel. If the insert was in the desired orientation (A), two fragments (11.5Kbp and 300bp) would be generated. If in the opposite orientation, two fragments of 10.5Kbp and 1.3Kbp would be generated. The presence of a band at ~300bp (and the absence of a band at 1.3Kbp) confirmed that the pCMV-NTR fragment had been cloned into the vector in the desired orientation.

Figure 12. Agarose gel electrophoresis of *Sca*I digested clone 4 (A) and HSV1716/CMV-NTR/GFP viral titres (B). Clone 4 (RL1.dCMV-NTR-GFP) was digested with *Sca*I, the digested DNA purified and 5µl electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel, to check its concentration. 80% confluent BHK cells were then co-transfected with 10µl HSV17$^+$ DNA and an appropriate volume of the remaining digested clone 4. The cells were incubated at 37°C for 3 days until cpe was evident. Recombinant viral plaques were picked under the fluorescent microscope, purified and a virus stock, named HSV1716/CMV-NTR/GFP, grown up. The cell-associated and cell-released fraction of the virus stock was titrated on BHK cells.

Figure 13. Growth kinetics of HSV17$^+$, HSV1716 and HSV1716/CMV-NTR/GFP in confluent BHK and 3T6 cells.

Confluent BHK and 3T6 cells were infected at a MOI of 0.1pfu/cell. Infected cells were harvested at 0, 4, 24, 48 and 72hrs post infection, sonicated and progeny virus titrated on BHK cell monolayers. All viruses replicated with similar kinetics in BHK cells (A); HSV1716 and HSV1716/CMV-NTR/GFP both failed to replicate efficiently in confluent 3T6 cells (B).

Figure 14. Western blot analysis of ICP34.5 expression in HSV17$^+$ and HSV1716/CMV-NTR/GFP infected BHK cells. BHK cells were infected with HSV17$^+$ and HSV1716/CMV-NTR/GFP at a MOI of 10pfu/cell. 16hrs post infection, the cells were harvested and protein extracts analysed using 10% SDS-PAGE in a Western blot using a polyclonal anti-ICP34.5 antibody. ICP34.5 was strongly expressed in HSV17$^+$ infected cells but was not expressed in HSV1716/CMV-NTR/GFP infected cells.

Figure 15. Western blot analysis of NTR expression in HSV1716/CMV-NTR/GFP infected cell lines. BHK, C8161, VM and 3T6 cells were infected with 10pfu/cell HSV1716/CMV-NTR/GFP, HSV17$^+$ or mock infected. 16hrs post infection, the cells were harvested and protein extracts analysed in a Western blot using a polyclonal NTR-specific antibody. Significant NTR expression was detected in all the HSV1716/CMV-NTR/GFP infected cells. No NTR expression was detected in the mock or HSV17$^+$ infected cells.

Figure 16. Effect of HSV1716/CMV-NTR/GFP and HSV1716-GFP with or without CB1954 (50μM) on confluent 3T6 cells. Confluent 3T6 cells in three wells of a 96-well plate were mock infected, infected with 1 or 10pfu/cell

HSV1716/CMV-NTR/GFP or infected with 1pfu/cell of HSV1716-GFP. 45 minutes later, infected cells were overlaid with media containing 50μM CB1954 or with media alone and incubated at 37°C. 24, 48, 72, 96, and 120hrs later, % cell survival was determined relative to that of mock infected cells without prodrug using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Figures shown represent the mean of 3 values +/- standard error of the mean.

Figure 17. Effect of HSV1716/CMV-NTR/GFP and HSV1716-GFP with or without CB1954 (50μM) on confluent C8161 cells. Confluent C8161 cells in three wells of a 96-well plate were mock infected, infected with 1 or 10pfu/cell HSV1716/CMV-NTR/GFP or infected with 1pfu/cell of HSV1716-GFP. 45 minutes later, infected cells were overlaid with media containing 50μM CB1954 or with media alone and incubated at 37°C. 24, 48 and 72hrs later, % cell survival was determined relative to that of mock infected cells without prodrug using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Figures shown represent the mean of 3 values +/- standard error of the mean.

Figure 18. Confluent 3T6 cells 72hrs post treatment with 10pfu/cell HSV1716/CMV-NTR/GFP (A), or 10pfu/cell HSV1716/CMV-NTR/GFP with 50μM CB1954 (B). The extent of cell death is significantly more pronounced in HSV1716/CMV-NTR/GFP infected cells overlaid with media containing 50μM CB1954 than in HSV1716/CMV-NTR/GFP infected cells overlaid with normal media. The extent of

cell death following infection of these cells with 10pfu/cell HSV1716, with or without CB1954, is comparable to that seen in A (data not shown). 50μM CB1954 alone has no effect on these cells.

Figure 19. Confluent C8161 cells 72hrs post treatment with 10pfu/cell HSV1716/CMV-NTR/GFP (A), or 10pfu/cell HSV1716/CMV-NTR/GFP with 50μM CB1954 (B). The extent of cell death is significantly more pronounced in HSV1716/CMV-NTR/GFP infected cells overlaid with media containing 50μM CB1954 than in HSV1716/CMV-NTR/GFP infected cells overlaid with normal media. The extent of cell death following infection of these cells with 10pfu/cell HSV1716, with or without CB1954, is comparable to that seen in A (data not shown). 50μM CB1954 alone has no effect on these cells.

Figure 20. Weight change (as a guide to health) in athymic nude mice with subcutaneous A2780 (xenograft) tumours injected intratumourally with HSV 1790. Group size = 3 mice per dose . A2780 xenografts at date of intratumoural injection (Day 0) are between 0.5 - 1mm in diameter. The xenografts have reached this size 12 days after injection with 10 million A2780 cells subcutaneously on the flank of female athymic nude mice.

Figure 21. Change in tumour volume over time in athymic nude mice with A2780 xenografts after intratumoural injection of HSV 1790.

Figure 22. Starting tumour sizes of mice.

Figure 23. Alterations in weight after treatment with CMV-ntr, CB1954 or a combination of both.

Figure 24. Change in tumour volume after treatment with CMV-ntr, CB1954 or a combination of both.

Figure 25. Starting tumour volume of each treatment group (see Table 2).

Figure 26. Weight (as a measurement of health) in athymic nude mice with A2780 xenograft treated with either HSV 1790, HSV 1716, CB 1954 or a combination of them.

Figure 27. Change in tumour volume of xenografts treated with the prodrug CB1954.

Figure 28. Changes in tumour volume in xenograft treated with 10^5 PFU HSV 1790 and CB1954.

Figure 29. Changes in tumour volume in xenografts treated with 10^6 PFU HSV 1790 and CB1954.

Figure 30. Changes in tumour volume in xenografts treated with 10^5 PFU HSV 1716 and CB1954.

Figure 31. Comparison of 10^5 PFU, 10^6 PFU HSV 1790 and 10^5 PFU HSV 1716.

Detailed Description of the Best Mode of the Invention

Specific details of the best mode contemplated by the inventors for carrying out the invention are set forth below, by way of example. It will be apparent to one skilled in the art that the present invention may be practiced without limitation to these specific details.

Example 1

Construction of plasmid RL1.dIRES-GFP

General Approach

Plasmid RL1.dIRES-GFP was generated in three stages, illustrated in Figure 1.

1. The DNA sequences containing the CMV IE promoter (pCMV), the NAT gene, the internal ribosome entry site (IRES), the GFP reporter gene and the SV40 polyadenylation sequences were excised from pNAT-IRES-GFP using *Nsi*I and *Ssp*I and purified.

2. The purified pCMV-NAT-IRES-GFP-PolyA DNA fragment was cloned into RL1.del to form a new plasmid designated RL1.dCMV-NAT-GFP.

3. The pCMV-NAT DNA sequences of RL1.dCMV-NAT-GFP were excised using *Xho*I and the remainder of the plasmid re-ligated to form a novel plasmid designated RL1.dIRES-GFP. This novel plasmid contained a multi-cloning site (all sites shown are unique) upstream of an IRES, the GFP gene and the SV40 polyA sequences all within the HSV-1 RL1 flanking sequences. Recombinant ICP34.5 null HSV-1,

expressing a gene of interest in the RL1 locus, can be generated by cloning the gene of interest (downstream of a suitable promoter) into the multi-cloning site and co-transfecting BHK cells with the plasmid and HSV-1 DNA. Recombinant virus expressing the target gene can be identified using GFP fluorescence.

Removal of the CMV promoter and noradrenaline transporter gene (pCMV-NAT) from RL1.dCMV-NAT-GFP, followed by re-ligation of the remainder of the plasmid, resulted in a novel plasmid (RL1.dIRES-GFP) containing a multi-cloning site (MCS), upstream of the encephalomyocarditis virus internal ribosome entry site (EMCV IRES), the GFP reporter gene and the SV40 PolyA sequences, all within RL1 flanking sequences. This novel arrangement of DNA sequences or 'smart cassette' allows ICP34.5 null HSV-1, expressing a gene of interest in the RL1 locus, to be easily generated by simply inserting the desired transgene (downstream of a suitable promoter) into the MCS and co-transfecting BHK cells with the plasmid and HSV-1 DNA. The IRES situated between the GFP gene and the MCS permits expression of two genes from the same promoter and so recombinant virus expressing the gene of interest also expresses GFP and can therefore be easily identified under a fluorescence microscope and purified.

Materials and Methods

1µg of RL1.del* was digested with 10units *Hpa*I (Promega) in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega) at 37^{0}C for 16hrs. The digested plasmid was then purified using the QIAquick PCR

purification kit (Qiagen), treated with 10 units of Calf Intestinal Phosphatase (Promega), in a suitable volume of 10x CIP buffer and nuclease free water for 4hrs at 37°C, before being purified again using a Qiaquick PCR purification kit. 5µl of the purified DNA was electrophoresed on a 1% agarose gel to check its concentration (Figure 2).

4 x 1µg of pNAT-IRES-GFP** was digested with 10 units of *Nsi*I and 10 units of *Ssp*I in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega) at 37°C for 16hrs. The reaction mixture was electrophoresed in a 1% agarose gel for 1hr at 110 volts. The 5.4Kbp DNA fragment consisting of the CMV IE promoter (pCMV), upstream of the noradrenaline transporter gene (NAT), the encephalomyocarditis virus internal ribosome entry site (IRES), the gene for green fluorescent protein (GFP) and the SV40 polyadenylation sequences (SV40 Poly A), was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit (Qiagen). The eluted DNA was blunt ended using 3 units Klenow Polymerase (Promega) in accordance with the manufacturers instructions and the DNA purified using a QIAquick PCR purification kit (Qiagen). 5µl of the purified DNA fragment was electrophoresed on a 1% agarose gel to check its concentration (Figure 3).

Ligation reactions were carried out in small eppendorf tubes containing 5 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega), nuclease free water (Promega) and various volumes of the *Hpa*I digested/CIP treated RL1.del and blunt ended pCMV-

NAT-IRES-GFP-SV40 Poly A DNA, at 16°C overnight. Competent JM109 bacterial cells (Promega) were then transformed with various aliqouts of the ligation reactions***. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened for inserts using *Afl*II (New England Biolabs) restriction enzyme analysis. Plasmid DNA containing the insert would produce two fragments of 4.8Kbp and 9.2Kbp following digestion with *Afl*II. Two clones (clone 5 and 8) contained the insert (Figure 4). The orientation of the insert in clone 5 (RL1.dCMV-NAT-GFP) was determined using *Xho*I restriction enzyme analysis (Figure 5).

To generate RL1.dIRES-GFP from clone 5, the CMV-NAT portion of the CMV-NAT-IRES-GFP-SV40 PolyA insert was removed by digesting 4 x 500ng of clone 5 with 10 units of *Xho*I in a suitable volume of buffer and water (Promega), overnight at 37°C. The digested DNA was electrophoresed on a 1% agarose gel at 110 volts for 1hr (Figure 6A). The 10.2Kbp fragment consisting of the IRES, the GFP gene, the SV40 PolyA sequences and RL1 flanking sequences in a pGEM3Zf(-) (Promega) backbone, was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit.

Ligation reactions were performed in small eppendorf tubes containing 100ng - 500ng purified DNA, 3 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega) and nuclease free water (Promega) overnight at 16°C. Competent JM109 bacterial cells (Promega) were then transformed with various aliquots of the ligation reactions***. Colonies formed on the plates

were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened using *Xho*I (Promega) restriction enzyme analysis. Colonies containing plasmid DNA from which CMV-NAT had been removed would produce one fragment of 10.2Kbp when digested with *Xho*I. Several positive clones were found, one was isolated, and a large-scale plasmid preparation undertaken using Promega's Wizard Plus Maxipreps kit. The large-scale plasmid preparation was checked by digesting with *Xho*I (Figure 6B). This plasmid DNA was subsequently named 'RL1.dIRES-GFP'.

Plasmid RL1.dIRES-GFP has been deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 03 September 2003 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03090303 in accordance with the provisions of the Budapest Treaty.

RL1.del

*RL1.del was provided by Dr.E.McKie and is the pGEM-3Zf(-) plasmid (Promega) into which has been cloned an HSV-1 fragment (123459-129403) consisting of the RL1 gene and its flanking sequences. The 477bp *Pfl*MI-*Bst*EII fragment of the RL1 gene (125292-125769) has been removed and replaced with a multi-cloning site (MCS) to form RL1.del.

pNAT-IRES-GFP

** pNAT-IRES-GFP was supplied by Dr. Marie Boyd (CRUK Beatson Laboratories) and is the pIRES2-EGFP plasmid (BD Biosciences Clontech) into which she has cloned the bovine noradrenaline transporter (NAT) gene (3.2Kbp), at the *Nhe*I and *Xho*I sites.

****Transformation of Bacterial Cells*

10µl of a glycerol *E.coli* stock was added to 10ml 2YT medium in a 20ml griener tube. This was placed in a 37°C shaking incubator for 16-24hrs until a saturated culture was obtained. 1ml of this culture was then added to 100ml of 2YT in a 500ml sterile glass bottle and placed in the 37°C shaking incubator for 3hrs. The bacterial cells were pelleted by centrifugation at 2,000rpm for 10 minutes (Beckman). The cells were then resuspended in $1/10^{th}$ volume of transformation and storage buffer (10mM $MgCl_2$, 10mM $Mg(SO)_4$, 10% (w/v) PEG 3,500, 5% (v/v) DMSO). The cells were placed on ice for between 10 minutes and 2hrs, after which time they were considered competent for transformation.

1-10µl of DNA was mixed with 100µl of competent bacteria in eppendorf tubes, and the tubes placed on ice for 30 minutes. After this, the samples were 'heat shocked' by incubating the tubes in a 42°C water bath for exactly 45 seconds before placing them on ice for a further 2 minutes. 1ml of L-Broth was added, the tube inverted 2-3 times, and the bacteria incubated for 1hr at 37°C. 100µl of the transformed bacteria was plated out onto L-broth agar plates containing 100µg/ml of the appropriate antibiotic (usually ampicillin or kanamycin). Plates

were allowed to dry at room temperature, before incubating in an inverted position at 37°C overnight.

Example 2

Generation of ICP34.5 null HSV-1 expressing a gene product of interest and GFP using plasmid RL1.dIRES-GFP.

General Approach

Generation of ICP34.5 null HSV-1 expressing a gene product of interest requires insertion of nucleotide sequence encoding the gene product (polypeptide) of interest, and often a desired promoter, at the MCS of RL1.dIRES.GFP followed by co-transfection of BHK cells with the linearised plasmid, containing the gene of interest, and HSV DNA. Following homologous recombination viral plaques expressing GFP are identified. Figure 7 illustrates the method steps involved.

Referring to Figure 7A plasmid DNA, containing the gene of interest and the desired promoter (X), is digested with restriction endonucleases to release the promoter/gene fragment.

The promoter/gene fragment is purified and cloned into the multi-cloning site (MCS) of RL1.dIRES.GFP forming a shuttle vector suitable for generating oncolytic HSV-1 (Figure 7B). This vector contains HSV-1 sequences that flank the essential RL1 gene but does not contain the RL1 gene. The plasmid also contains the gene for Green Fluorescent Protein (GFP) downstream of an internal

ribosome entry site (IRES). The IRES permits expression of both the gene of interest and the GFP gene from the same upstream promoter.

BHK cells are then co-transfected with linearised RL1.dIRES.GFP, now containing the gene of interest, and HSV-1 DNA (Figure 7C). Following homologous recombination, designer virus, expresssing the gene of interest and GFP, is generated and can be distinguished from wild type virus (also generated but not expressing GFP) under a fluorescence microscope.

Viral plaques, expressing GFP (and hence the gene of interest), are picked under the fluorescence microscope and purified until all wild-type HSV-1 has been removed. The recombinant HSV-1 is considered 100% pure when all the viral plaques are expressing GFP (Figure 7D).

Once the recombinant virus is completely pure, an isolated plaque is picked and a highly concentrated stock is grown and titrated (Figure 7E). Oncolytic HSV-1, expressing a gene product of interest from a selected promoter, is then ready for characterisation and in vitro examination of its tumour killing potential.

Materials and Methods

To generate recombinant ICP34.5 null HSV-1 expressing a gene of interest and GFP, requires the gene of interes, and often a suitable promoter, to be cloned into the MCS of RL1.dIRES-GFP in the forward orientation with respect to the GFP gene in this plasmid. Once this has been

achieved the plasmid is linearised (i.e. digested with a restriction enzyme that cuts only once, usually *Ssp*I or *Sca*I) in an irrelevant region. 80% confluent BHK cells in 60 mm petri dishes are then co-transfected with HSV-1 DNA and linearised plasmid DNA as described below.

To generate replication restricted HSV-1, expressing the gene of interest and GFP, the gene of interest must be cloned into RL1.dIRES-GFP downstream of a suitable promoter (e.g. CMV IE). The promoter is required upstream of the gene of interest for the production of a bicistronic mRNA transcript. The IRES sequence between the two open reading frames in the transcript functions as a ribosome binding site for efficient cap-independent internal initiation of translation. The design enables coupled transcription of both the gene of interest and GFP, followed by cap-dependent initiation of translation of the first gene (gene of interest) and IRES-directed, cap-independent translation of GFP. Co-ordinate gene expression is thus ensured in this configuration.

Co-Transfection of Virus and Plasmid DNA by $CaPO_4$ and DMSO Boost

HSV-1 (17^+) DNA and 0.1-1µg linearized SMART cassette containing the gene and promoter of interest is pipetted into 1.5ml eppendorf tubes containing 1µl of calf thymus DNA (10µg/ml) and an appropriate volume of distilled water to give a final volume of 165µl. The solutions are very gently mixed using a 200µl pipette tip. 388µl of HEBS, pH 7.5, (130mM NaCl, 4.9mM KCl, 1.6mM Na_2HPO_4, 5.5mM D-glucose, 21mM HEPES) is then added, the solution mixed, before adding 26.5µl of 2M $CaCl_2$ dropwise and flicking the

eppendorf tube two or three times. The samples are left at room temperature for 10-15 minutes then added dropwise to 80% confluent BHK's in 60mm petri dishes from which the medium has been removed. Following incubation at 37°C for 45 minutes, the cells are overlaid with 5ml of ETC10 and incubated at 37°C. Three to four hours later, the media is removed and the plates washed with ETC10. For exactly 4 minutes, the cells are overlaid with 1ml 25% (v/v) DMSO in HEBS at room temperature. After the 4 minutes, the cells are immediately washed three times with 5ml ETC10 before overlaying with 5ml of ETC10 and returning to the incubator. The following day, fresh medium is added to the cells. Two days later, when cpe is evident, cells are scraped into the medium, transferred to small bijoux and sonicated thoroughly. The sample is then stored at -70°C until required (see section below on plaque purification).

N.B. The volume of virus DNA to add is determined by undertaking the above procedure without plasmid DNA, using a range of virus DNA volumes and choosing the volume that gives the greatest number of viral plaques on the BHK monolayer after 2 or 3 days.

Plaque Purification

Sonicated samples from co-transfection plates are thawed and serially diluted 10 fold in ETC10. 100µl from neat to the 10^5 dilution is plated out on confluent BHK's in 60 mm petri dishes from which the media has been removed. After 45 minutes incubation at 37°C, the cells are overlaid with 5ml EMC10 and incubated at 37°C for 48hrs. The plates are then checked for the presence of viral

plaques and those dishes with the fewest, most separated plaques are placed under a fluorescent stereomicroscope. Recombinant virus, designed to express the green fluorescent protein (GFP) in addition to the gene of interest, can clearly be distinguished from wild type virus using a GFP filter. Fluorescent plaques are picked using a 20µl pipette and placed (including the tip) into an eppendorf tube containing 1ml ETC10. The sample is thoroughly sonicated before making serial 10 fold dilutions in ETC10 and repeating the above purification procedure. The process is repeated typically 3-4 times until every plaque on the BHK monolayer is fluorescent. Once this has been achieved, 50µl of this sample is used to infect BHK cells in roller bottles, in 50ml ETC10, and a virus stock grown.

Tissue Culture Media

BHK21/C13 cells are grown in Eagle's medium (Gibco) supplemented with 10% newborn calf serum (Gibco) and 10% (v/v) tryptose phosphate broth. This is referred to as ETC10. For virus titrations and plaque purification, EMC10 (Eagles medium containing 1.5% methylcellulose and 10% newborn calf serum) is used to overlay the cells.

Example 3

Construction of HSV1716/CMV-NTR/GFP

General Approach

HSV1716/CMV-NTR/GFP was generated by cloning a 1.6Kbp *Bam*HI fragment from pPS949[10], consisting of the *E.coli*

nitroreductase (NTR) gene downstream of the CMV IE promoter (pCMV), into the MCS of the RL1.dIRES-GFP smart cassette, in the forward orientation with respect to the GFP gene in RL1.dIRES-GFP (Figure 8). The resultant plasmid, named RL1.dCMV-NTR-GFP, was then linearised and recombinant virus generated and purified as described above. The plasmid pPS949 (referred to as 'pxLNC-ntr' in Ref 10) containing the NTR gene downstream of the CMV IE promoter (pCMV-NTR) in a pLNCX (Clontech) backbone, was a kind gift from Professor Lawrence Young, University of Birmingham, UK.

Materials and Methods

4 x 1µg of pPS949 was digested with 10 units of *Bam*HI (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37^{0}C for 16hrs. The reaction mixture was electrophoresed in a 1% agarose gel for 1hr at 110 volts. The 1.6Kbp DNA fragment consisting of the CMV promoter upstream of the NTR gene (pCMV-NTR), was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit (Qiagen). 5µl of the purified DNA fragment was electrophoresed on a 1% agarose gel to check its concentration (Figure 9).

2µg of the RL1.dIRES-GFP smart cassette was then digested with 15 units of *Bgl*II (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37^{0}C for 16hrs. The digested plasmid was then purified using the QIAquick PCR purification kit (Qiagen), treated with 10 units of Calf Intestinal Phosphatase (Promega),

in a suitable volume of 10x CIP buffer and nuclease free water for 4hrs at 37°C, before being purified again using the Qiaquick PCR purification kit. 5µl of the purified DNA was electrophoresed on a 1% agarose gel to check its concentration (Figure 10).

Ligation reactions were carried out in small eppendorf tubes containing 5 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega), nuclease free water (Promega) and various volumes of the *BglII* digested/CIP treated RL1.dIRES-GFP smart cassette and pCMV-NTR (*Bam*HI ends), at 16°C overnight. Competent JM109 bacterial cells (Promega) were then transformed with various aliqouts of the ligation reactions. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened for inserts using *BglII/XhoI* (Promega) restriction enzyme analysis. RL1.dIRES-GFP plasmid DNA containing the pCMV-NTR insert in the correct orientation would produce two fragments of 11.5Kbp and 300bp following digestion with *BglII* and *XhoI*. One clone (clone 4) was found to contain the insert in the correct orientation (Figure 11). This plasmid was named 'RL1.dCMV-NTR-GFP'.

0.1-1µg of RL1.dCMV-NTR-GFP was linearized by digesting with 10 units of *ScaI* (Promega), in a suitable volume of 10x buffer (Promega) and nuclease free water (Promega), at 37°C for 16hrs. A sample (5µl) of the digested DNA was electrophoresed on a 1% agarose gel for 1hr at 110 volts to check that it had been linearized. 80% confluent BHK cells were then co-transfected with a suitable volume of the remaining linearised DNA and HSV-1 DNA. Recombinant

HSV-1, expressing GFP (and hence NTR), was identified and purified using a fluorescent microscope and a virus stock, named HSV1716/CMV-NTR/GFP, was grown and titrated on BHK cells (Figure 12).

HSV1716/CMV-NTR/GFP has been deposited in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 05 November 2003 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 03110501 in accordance with the provisions of the Budapest Treaty.

HSV1716/CMV-NTR/GFP Cell Killing

HSV1716/CMV-NTR/GFP replicates with almost identical kinetics to HSV1716 in BHK cells and 3T6 cells. BHK cells support the replication of ICP34.5 null HSV while confluent 3T6 cells do not. Figure 13 shows that HSV1716/CMV-NTR/GFP will replicate as well as HSV1716 in permissive cell lines and that the introduction of exogenous genes, e.g. NTR and GFP, has not reduced the oncolytic potential of the ICP34.5 null HSV. The fact that HSV1716/CMV-NTR/GFP fails to replicate in 3T6 cells also indicates that this recombinant HSV is an ICP34.5 null mutant.

Figure 14 is a Western blot demonstrating that no ICP34.5 polypeptide is expressed from HSV1716/CMV-NTR/GFP, and that the virus is thus useful as a gene therapy vector.

Figure 15 is another Western blot demonstrating expression of NTR in a variety of cell lines infected with HSV1716/CMV-NTR/GFP, including a human malignant melanoma cell line (C8161) and confluent 3T6 cells in which ICP34.5 null HSV does not replicate. Expression of NTR in confluent 3T6 cells, following infection with HSV1716/CMV-NTR/GFP, is encouraging as it demonstrates that replication of this ICP34.5 null mutant is not required for expression of the prodrug-activating gene (i.e. NTR). Some tumour cells *in vivo* will not support the replication of ICP34.5 null HSV and as such, will not be killed with HSV1716.

Figure 16 shows the results from a cytotoxicity assay performed in confluent 3T6 cells. Infecting confluent 3T6 cells with an ICP34.5 null mutant (HSV1716/CMV-NTR/GFP), at a multiplicity of infection (MOI) of 1 plaque forming units (pfu)/cell, does not result in any significant cell death, neither does separate incubation of the cells with 50μM CB1954. However, significant cell death is evident 72hrs post infection with 1pfu/cell HSV1716/CMV-NTR/GFP when 50μM CB1954 is included in the growth medium. This clearly demonstrates that when there is no replication of the virus, substantial cell death is still possible from virus directed enzyme prodrug therapy (VDEPT).

Infecting confluent 3T6 cells with an ICP34.5 null mutant at a MOI of 10pfu/cell will result in cell death, by a mechanism known as 'viral antigen overload'. However, the level of cell killing is even more pronounced

(approximately 20% more), when 50μM CB1954 is included in the growth medium.

A similar cytotoxicity assay was performed in human C8161 melanoma cells, the results are set out in Figure 17. Unlike confluent 3T6 cells, C8161 cells do support the replication of ICP34.5 null HSV. Therefore, cell death will occur following infection of the cells with ICP34.5 null HSV, at 1pfu/cell. However, when CB1954 is included in the overlay of HSV1716/CMV-NTR/GFP infected cells, the cells are killed more efficiently and more quickly. No enhanced cell killing is evident when CB1954 is included in the overlay of cells infected with HSV1716-GFP. These results demonstrate that enhanced cell killing is possible in human tumour cells.

Cell culture images for the cytotoxicity assays performed in confluent 3T6 and human C8161 melanoma cells are shown in Figures 18 and 19.

Example 4 - In vivo evaluation of the anti-tumour activity of a selectively replication competent herpes simplex virus in combination with enzyme pro-drug therapy.

The anti-tumour activity of a selectively replication competent herpes simplex virus in combination with an enzyme prodrug therapy approach in appropriate animal models *in vivo* was investigated.

The parental virus, HSV 1716 is a selectively replication competent mutant of the herpes simplex virus 1 (HSV 1)

which lacks both copies of the RL1 gene that encodes the protein ICP 34.5. This protein is a specific determinant of virulence. The function of this protein has been described at length elsewhere[12]. The virus can grow only in cells that have a high level of functional PCNA. High levels of PCNA are found only in cells that are dividing such as tumour cells and not normal differentiated cells.

It has already been shown that HSV 1716 can achieve selective tumour cell killing with minimal toxicity and improved survival times in a number of animal models[13]. Initial phase 1 clinical trials using HSV1716 virus in patients has also meet with some success[14, 15].

Although HSV1716 selectivity replicates in tumour reducing the tumour bulk by cell lysis the inventors did not anticipate HSV1716 to lytically replicate in all cells in the tumour due to the heterogeneity of the cell type and growth state.

In order to enhance the efficacy of the tumour cell killing - hence kill the entire tumour - the invenots have constructed a derivative HSV1790 of HSV1716 that expresses the E.Coli nitroreductase gene (*ntr*) under the control of a CMV early promoter (see example 3 above). The enzyme *ntr* converts the inactive prodrug CB1954 to a functional cytotoxic alkylating agent that kills both dividing and non-dividing cells by apoptosis. This active drug is diffusible and membrane permeable resulting in an efficient bystander effect, i.e. wherein the activated drug may have an effect on surrounding cells.

As the prodrug will only be converted to its active form in the tumour which has been infected with *ntr* expressing virus, toxicity to normal cells is avoided hence improving the therapeutic index following systemic delivery of this compound.

Initial *in vitro* experiments using this combination have already shown enhanced cell kill using this virus in combination with CB1954 in a number of cell lines.

This example further evaluates this combination approach in vivo in appropriate animal models.

Results

Months 1 - 3

Months 1 - 3 were taken up mainly by *in vitro* work. During this time period high titre, sterile virus stock was generated for use in the xenograft models.

Xenograft models were also generated in athymic nude mice using the cell line A2780, a human ovarian epithelial carcinoma line initially derived from a tumour sample from an untreated patient (European Collection of Cell Cultures (ECACC) CAMR, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom, accession number 93112520).

Generation of a gliomal xenograft model was attempted using 2 gliomal lines that were available in house, LN-18 and U373MG. There are reports in the literature of both being successfully grown as xenografts in athymic mice.

However, as shown in the table below the inventors failed to see any xenograft growth up to 28 days after injection with 5 million cells subcutaneously.

Table 1

Cell Line	No of cells injected per mice	Number of mice	Presence of xenograft 28 days after cell injection
LN 18	5 million	5	0/5
U373 MG	5 million	5	0/5

A2780 tumour take

As reported previously A2780 have a Take Rate of approximately 50 % - that is 50 % of mice that are injected with 5 million cells per flank subcutaneously will develop xenografts. When the number of cells injected was increased to 10 million or more an increase in take rate of approximately 15 - 25 % was seen, giving an overall take rate of 65 - 75 %.

Thus increasing the number of gliomal cells injected may increase the take rate of these cell lines

Dose response to the HSV 1790 virus

Before the mice can be treated with a combination of virus and prodrug, first one must carry out experiments

to make informed decisions about how much of the virus, and the prodrug to give.

A dose response experiment will allow one to find both the most appropriate does of the virus to use in the experiments and the maximum tolerated dose (MTD) of the virus, that is, the largest amount of the virus that can be given to a single mouse without adverse side effects. Small groups of tumour bearing mice are given a small dose of virus. Assuming they do not have any adverse effects another group is given a larger dose of virus. This continues until either the mice start to suffer ill effects or we reach a maximum dose.

The maximum amount of virus that can be intratumourally injected is 100 µl, hence the maximum dose from our current stock is 10^9 PFU per injection.

Figure 20 shows the weight change in the mice after injection with a variety of doses of virus. Weight is a good indicator of the animals overall health. Any loss of weight signifies that the treatment is not being well tolerated. Where an animal loses more than 20% of its initial body weight it was sacrificed immediately.

A dose of 10^9 PFU of the HSV 1790 virus is not tolerated by these mice, they rapidly lost body weight and were sacrificed at Day 3 post injection. Doses of 10^8 PFU or less were better tolerated, the mice initially lost weight in the days following injection but quickly recovered to approximately their initial body weights.

It should be pointed out that as the experiment progresses the animals appear to be increasing in weight. This is almost certainly due to the fact that it is total body weight that is measured, which includes the weight of any tumour that is forming.

Response of the tumour to HSV 1790 treatment

Tumour volume was measured daily after intratumoural injection of the HSV 1790 virus to look for any growth delay or regression of the tumours.

Figure 21 shows the change in tumour volume as measured over a period of 100 days. If the tumour was injected with PBS only as a control the tumour increased in size rapidly and by approximately Day 13 post injection the tumours had become too large and the animals had to be sacrificed.

Treatment with all doses of virus appeared to delay the growth of the tumour to some degree. Doses of 10^5 PFU increased the longevity of the mice by approximately 12 days while mice injected with 10^6 PFU virus tumours survived for an extra 23 days compared to the control group before the tumours became prohibitively large. Perhaps surprisingly the group of mice injected with 10^8 PFU of virus survived only slightly longer than the control group. It is possible there were a large number of non infectious particles or that sheer number of particles caused the cells which the virus would have grown in to be killed.

The group of mice treated with 10^7 PFU of virus survived the longest and indeed two out of three of the mice did not have any visible signs of tumour when sacrificed at day 100.

Naked DNA Experiments

In order to check the alterations in tumour growth are due to the virus itself and not a result of the CMV-ntr plasmid DNA that had been introduced to the HSV 1716 virus, an experiment was set up looking at the effect of the CMV-ntr plasmid DNA alone and in combination with the prodrug CB1954.

Mice were randomised into treatment groups of 6 animals each when tumour diameters are approximately 5mm (this is Day 0). Figure 22 shows the starting tumour diameters for the mice used in this experiment. Two groups of mice were administered CMV-ntr plasmid by direct intratumoural injection at a dose of 0.2mg DNA per injection. One of these groups was then administered with a single dose of 80mg/kg of CB1954 on Day 2 by intra-peritoneal injection. The third group of mice had a single administration of CB1954 (80mg/kg) by intra-peritoneal injection on Day 2 following intratumoural injection of saline control at Day 0. Animals were weighed daily (Figure 23) and daily caliper measurements performed until the tumour sizes were in the region of 20 mm by 20mm. Tumour volumes were estimated from these measurements (volume = d3 x 6) (Figure 24). In addition any toxicity from these administered agents was determined. On the basis of these experiments the inventors determined that neither the

CMV-ntr alone, CB1954 alone or the combination of both CMV-ntr and CB1954, has any anti - tumour activity as determined by tumour regression in this model system (Figure 24).

Scheduling Experiment

Previous dose response experiments have shown that doses of less than 10^8 PFU virus per mouse do not appear to have any adverse effect of the animals health.

A dose of 10^7 PFU virus per mouse resulted in a great reduction in tumour growth, indeed after 100 days two out of three of the mice in the group had no visible tumour. This is very encouraging - the virus only at high doses may be enough to delay growth or cause tumour regression.

However to look at the effect of a combination of the virus and the prodrug CB 1954 a lower dose of the virus was studied - if the treatment of the virus alone results in growth delay for such a long period one would be unable to ascertain the addition or synergistic effects of the prodrug.

The inventors proceeded to investigate two doses of the virus in combination with CB1954. The doses selected were 10^5 PFU and 10^6 PFU. Both these doses caused some tumour growth delay in earlier experiments.

The prodrug is given as an 80mg/kg intra-peritoneal injection, after dissolving the powdered form in 10% acetone and then making up the volume with peanut oil.

Another factor that determines how well the drug will work - hence how much tumour growth delay or regression is seen - is when the drug is actually given. As the prodrug will only be converted to an active substrate in the presence of NTR it was considered that the virus containing the NTR will have to been given first. It was also considered that if the virus is given time to replicate and produce more NTR then the prodrug may have a more pronounced effect.

To discover the optimal doses of both the virus and the drug and the optimal times of these treatments a scheduling experiment was set up.

Mice were randomized into treatment groups (treatment regimes shown in Table 2) of 3 animals when tumour diameters were approximately 5mm (tumour volume 0.5 - 1.5 mm^3). Figure 25 shows the starting tumour volumes of each of the groups.

Table 2: Treatment groups

1	10^5 HSV 1790 + drug (Day 2) + Drug (Day10)
2	10^5 HSV 1790 + drug (Day 2) + vehicle (Day 10)
3	10^5 HSV 1790 + vehicle (Day 2) + drug (Day 10)
4	10^5 HSV 1790 + vehicle (Day 2) + vehicle (Day 10)
5	No virus + drug (Day 2) + drug (Day 10)
6	No virus + drug (Day 2) + vehicle (Day 10)
7	No virus + vehicle (Day 2) + drug (Day 10)
8	No virus + vehicle (Day 2) + vehicle (Day 10)
9	10^6 HSV 1790 + vehicle (Day 2) + vehicle (Day 10)
10	10^6 HSV 1790 + drug (Day 2) + vehicle (Day 10)
11	10^6 HSV 1790 + drug (Day 2) + drug (Day 10)
12	10^6 HSV 1790 + drug (Day 2) + drug (Day 10) + drug (Day 15)
13	No virus + drug (Day 2) + drug (Day 10)
14	No virus + vehicle (Day 2) + vehicle (Day 10)
15	10^5 HSV 1716 + drug (Day 2) + drug (Day 10)
16	10^5 HSV 1716 + vehicle (Day 2) + vehicle (Day 10)
17	10^5 HSV 1716 + drug (Day 2) + vehicle (Day 10)
18	10^5 HSV 1716 + vehicle (Day 2) + drug (Day 10)

The treatment groups were administered with a single direct intratumoural injection of the virus and dose determined for that group. The virus was diluted PBS + 10% serum. 'No virus' control groups received an intratumoural injection of 100µl of PBS + 10% serum. This day was designated as Experimental Day 0.

The intratumoural injections did not appear to have any adverse effects on the mice. Some tumours bleed slightly after injection but not to a great degree. The animals did not lose body weight (Figure 26) and their behaviour did not appear to alter. In all the tumours that bleed slightly, the following day the healing process had begun and within 3 - 5 days there was little evidence of any puncture wound on any tumour.

Injections of CB1954 were given to the appropriate groups at days 2, 10 and 15. A dose of 80mg/kg - the equivalent of approx. 2mg per mouse - was given. The powdered form of the CB1954 drug (from Sigma) was dissolved in acetone to 10% of the final volume (10µl per 2mg). The volume was then made up to 2mg CB1954 in 100µl using peanut oil. A syringe was used to mix the drug as peanut oil is thick and viscous. The drug was made up fresh every time.

The appropriate groups were then injected intra-peritoneal with this solution. Control groups which were not receiving drug were injected intra-peritoneal with a 100µl solution of 10% acetone in peanut oil.

No swelling or irritation at the site of injection was noted on any of the mice either at time of injection or at any later time point. The mice appeared slightly lethargic for a short period after the injection but did not lose any body weight (Figure 26) or show signs of lethargy the following day.

No virus + CB1954 prodrug

Groups 5,6,7,8, 13 & 14 looked at the effect of prodrug alone on tumour growth. Figure 27 shows that there is little effect on tumour growth when CB1954 is given alone.

10^5 PFU virus +/- CB1954 prodrug

10^5 PFU virus was given at Day 0 followed by either prodrug or vehicle at Days 2 and 10.

As can be seen from the graph in Figure 28 tumours treated with either virus only or virus and prodrug did not grow as large as the untreated tumour. The tumour treated with the virus grew only to approximately half the size of the untreated control.

Treatment with virus and prodrug resulted in tumours which grew to only approx 2 - 3 mm^3 in volume. This is significantly less than the untreated tumours which grew in size to approx 20 mm^3.

10^6 PFU virus +/- CB1954 prodrug

Figure 29 shows the changes in tumour volume over time after treatment with a higher dose of virus, 10^6 PFU per injection, in combination with the prodrug, given as described in Table 2. As with the lower virus dose, treatment with either virus only, or in combination with CB1954, results in significantly smaller tumours compared to the untreated controls.

HSV 1716 virus in combination with CB1954 prodrug

The parental strain of the virus, which has not been engineered to contain the CMV-ntr DNA was examined for its effects on tumour growth delay. This virus does have an oncolytic effect, however it doesn't contain the NTR gene needed to convert the inactive prodrug into its active metabolite. Therefore one would not expect any additional or synergistic effects when the prodrug is added in combination with the virus. Figure 30 shows the results of this experiment.

The combination of the virus and the prodrug appeared to produce some reduction in tumour growth compared to the untreated control tumours.

The groups used in these results contained only 2 or 3 animals. The animals used were also older and their tumours had taken longer to grow than those used in previous experiments. Hence it is possible that repeating the experiment with a larger number, with younger mice or quicker forming tumours may result in a more marked growth delay after treatment with the HSV 1716 virus.

Comparison of HSV 1790 (at 10^5 and 10^6) and HSV 1716 in combination with CB1954 prodrug

Figure 31 shows a comparison between the two doses of the HSV 1790 virus in combination with the prodrug and the HSV 1716 prodrug combination. The parental virus HSV 1716 shows some growth delay in comparison with the untreated control. We would assume that this growth delay is due to the oncolytic effect of the virus as the NTR gene is not present to alter the inactive prodrug into its active form.

Tumour growth is reduced further when the tumour is treated with the HSV 1790 virus containing the NTR gene. This appears to be dose dependent - the higher dose of the virus results in a greater growth delay than the lower dose.

In conclusion it would appear from these results that indeed the HSV 1790 virus used in combination with the prodrug CB1954 results in growth delay in the model system examined. Giving both virus and drug in combination has a greater effect than given either alone.

It appears that the timing at which the prodrug is given after virus treatment is important. When CB1954 was given soon after viral injection (Day 2 post viral injection) tumour growth was not delayed as much as if the drug was given at a later date (Day 10). It may be that given at Day 2 the drug killed the cells that were supporting viral growth and replication and actually reduced the oncolytic effect of the virus.

By day 10 the virus may have replicated and killed as many cells by oncolysis as possible. It is anticipated that due to heterogeneity of the cell type and growth state that all the cells within a tumour would not be susceptible to lysis by the virus. The drug then comes in and 'mops up' by killing any cells that are supporting viral growth (hence containing the NTR gene) but were not susceptible to oncolysis. As the active drug is diffusible and membrane permeable it may have a bystander effect - killing not only the cells infected with the virus but also its near neighbours.

Example 5 - Construction of HSV1716 variants expressing siRNA

General Strategy

A plasmid that contains the siRNA construct designed to target expression of the SCCRO gene (SEQ ID No. 1) and designated 339i was provided by Dr Bhuv Singh, MSKCC, New York. A plasmid encoding a control siRNA (SEQ ID No 2), designated Coni, was also provided.

DNA sequences for the two constructs are as follows:

339isiRNA (SEQ ID No. 1):

gatcCCCGTTCAGAGCAGCAACACAGTTCAAGAGACTGTGTTGCTGCTCTGA
ACTTTTTGGAAA

ConisiRNA (SEQ ID No. 2):

gatcCCCCGTCTACCTACACTCCCTCTTCAAGAGAGAGGGAGTGTAGGTAGA
CGTTTTTA

Both siRNA constructs were in the vector pSNRG and their expression is driven by the RNA polIII H1 promoter. RNA polIII only transcribes short RNA molecules and the H1 promoter would be insufficient to drive expression of IRES-gfp from the normal recombinant virus producing shuttle vector RL1-del.IRES.gfp so an alternative cloning strategy was adopted.

A cassette was constructed in the following manner. The 1.3kbp blunt-ended EcoRI/AflII fragment that contains the PGK promoter/GFP gene was obtained by restriction digestion followed by Klenow treatment from the vector pSNRG and cloned into the RL1-del vector cut with the restriction enzyme NruI that generates blunt ends. Successful insertion of the PGK/GFP DNA was confirmed by BamHI digestion and the orientation of the inserted DNA identified using the unique XhoI site in RL1-del and the BsrGI site at the 3' end of PGK/GFP. Plasmids with PGK/GFP in both forward and reverse orientation were obtained and the plasmids were designated RL1-dPGK/GFPfor and RL1-dPGK/GFPrev. Expression of GFP was confirmed in BHK cells transfected with the forward and reverse orientation plasmids.

Thus, sequences of interest along with their own promoters (in this arrangement it is preferred that a different promoter is used to drive transcription of the

nucleotide sequence of interest and marker) can then be cloned into either RL1-dPGK/GFPfor or RL1-dPGK/GFPrev in either orientation using the remaining unique BglII, XhoI or HpaI unique restriction enzyme sites. The resulting plasmid can be used to derive recombinant HSV in which the marker GFP gene and the gene of interest are expressed independently from their own promoters

Materials and methods

In the pSNRG plasmid and adjacent to the H1/siRNA coding sequence is a green fluorescent protein (gfp) expression cassette comprising the gfp gene with a Phosphoglycerokinase (PGK) promoter. Using the restriction enzymes HindIII and AflII sequentially, the 1.6kbp DNA fragment that contains the H1/siRNA and PGK/EGFP expression cassettes were excised from their Coni and 339i plasmids. The 1.6kbp DNA fragment was purified from a 1% agarose gel and blunt-ended by incubation with Klenow DNA polymerase for 30 minutes at 30°C. The blunt-ended fragment was ligated into the RL1-del shuttle vector which had been digested with the restriction enzyme Nru1 that produces a blunt-ended cut. Before ligation the Nru1-cut RL1-del was gel purified and phosphatase-treated using Calf Intestinal Alkaline Phosphatase. After an overnight ligation with either the blunt-ended 339i or Coni DNA fragments with the blunt-ended RL1-del plasmid, the reaction mix was used to transform DH5alpha cells and these were plated-out on LB amp plates. After overnight incubation at 37°C, individual clones from each of the LB amp plates were grown overnight in 3ml of LB broth and plasmid DNA extracted.

To screen for recombinants, plasmids were initially digested with BamHI, as insertion of the H1/siRNA and PGK/gfp cassette increases the size of the RL1 BamHI fragment in the plasmid from 5.4kbp to 7.0kbp. For both Coni and 339i ligations 1/24 clones screened demonstrated a 7.0kbp BamHI fragment and the presence of the H1/siRNA and PGK/EGFP cassette in these plasmids was confirmed by EcoR1, EcoR1/HindIII and EcoR1/SalI digests, the inserted H1/siRNA and PGK/EGFP cassette introduces a novel EcoR1 site into the RL1-del vector.

From a glycerol stock of the positive 339i and Coni clones, additional plasmid was prepared and used to transfect BHK cells. Fifty microlitres (50µl) of plasmid was mixed with 6µl lipofectamine 2000 in a final volume of 100µl serum free medium and used to transfect BHK cells plated out on a 13mm glass coverslip in a 24-well plate. After 48hrs of transfection the cells were washed once in PBS, incubated for 2hrs in 4% paraformaldehyde, washed once more in PBS and mounted on microscope slides using Vectashield. The presence of c5% gfp-positive cells following transfection with the RL1-del/339i and RL1-del/Coni plasmids confirmed the presence of the PGK/GFP cassette.

The RL1-del/339i and RL1-del/Coni plasmids were linearized using either of the restriction enzymes ScaI and XmnI and the linearized plasmid was used along with viral DNA to transfect BHK cells plated out to c80% confluency in 60mm dishes. To 100µl of linearized plasmid or undigested circular plasmid, 50µl of HSV-1 strain 17+

DNA was added along with 20µl lipofectamine 2000 in a final volume of 500µl serum free medium and the mix added to the BHK cells. After 4hrs of transfection, the cells were shocked with 25% DMSO in HBSS for exactly 4 minutes, washed x3 with medium and returned to 37°C incubation in 5ml of medium for 48hrs. Viral cpe was evident after 48hrs and the cells and medium were harvested together, sonicated and stored at −80°C. Undiluted medium/cells and 4x 10-fold dilutions were plated out on BHK cells and, after 48hrs, viral plaques were examined by fluorescent microscopy for gfp expression. On the undiluted plate from cells transfected with XmnI-linearized plasmid >100 gfp-positive plaques were observed for both Coni and 339i indicating a high degree of recombination. Interestingly, recombination, but at a lower frequency (c50 plaques/plate), was observed for the transfected circular plasmid but recombination with the ScaI-linearized plasmid was very low (<5 plaques/plate).

Using the highest dilution at which gfp-positive plaques were clearly visible (the PGK/GFP cassette gave a very strong fluorescent signal), two plaques each of Coni and 339i viruses were picked using a sterile pipette tip, placed in 1ml medium, sonicated for 1 minute and stored at −80°C. Plaques were then subjected to 6 rounds of plaque purification, after the 6th round no wild type, non-gfp expressing plaques were visible and 6 plaques each of Coni or 339i virus were picked for Southern blotting.

Each of the six plaques of Coni and 339i virus was used to infect a T175 flask of Vero cells, after 72hrs of

infection virus was harvested and titred. For 3 each of the Coni and 339i viruses that gave the highest titres 0.5ml was used to infect a second T175 flask for 24hrs. Viral DNA was then harvested from each of the 6 flasks. The BamHI-digested viral DNA was Southern blotted with the Alu/Rsa RL1 probe and the band pattern compared to wild type and HSV1716 DNA digested also with BamHI. A novel c6kbp band, consistent with the insertion of the 1.6kbp H1/siRNA and PGK/GFP cassette in the RL1 locus, was clearly visible in all six viral isolates and no wild type bands were detected. Stocks of the Coni and 339i viruses that gave the strongest signal on Southern blotting were produced.

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